UNIFORMED SERVICES UNIVERSITY
OF THE
HEALTH SCIENCES
SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814

GRADUATE AND
CONTINUING EDUCATION

TEACHING HOSPITALS
WALTER REED ARMY MEDICAL CENTER
NAVAL HOSPITAL, BETHESDA
MALCOLM GROW AIR FORCE MEDICAL CENTER
WILFORD HALL AIR FORCE MEDICAL CENTER

APPROVAL SHEET

Title of Thesis: A Comparative Study of the Metabolism of 6-Methylbenzo[A]pyrene and Benzo[A]pyrene By Rat Liver Microsomes

Name of Candidate: Ms. Karen L. Hamernik
Doctor of Philosophy Degree
February 24, 1984

Thesis and Abstract Approved:

Committee Chairperson	Date: Feb. 24, 1984
Committee Member	Date: 2/24/84
Committee Member	Date: 2/24/84
Committee Member	Date: 2/24/84
Committee Member	Date: 2/24/84

ABSTRACT

Title of Dissertation: A Comparative Study of the Metabolism of 6-Methylbenzo[a]pyrene and Benzo[a]pyrene by Rat Liver Microsomes

Karen Lee Hamernik, Doctor of Philosophy, 1984

Dissertation directed by: Shen K. Yang, Ph.D., Professor, Department of Pharmacology

In several types of carcinogenicity studies, 6-methylbenzo[a]-pyrene (6-MBaP) was found to be moderately active as a tumorigen, although with potency less than that of benzo[a]pyrene (BaP). BaP is metabolically converted to an ultimate carcinogen, the BaP (+)-trans-7,8-diol 9,10-epoxide, by an activation pathway that proceeds via a (-)-trans-7,8-diol metabolite intermediate. It has not been established if 6-MBaP is activated by a similar pathway, and alternative mechanisms for the bioactivation of 6-MBaP have been proposed which involve reactive species other than a diol epoxide. The major goal of this study was to determine the effect of methyl substitution at position six on the in vitro metabolism of 6-MBaP relative to that of BaP in order to better understand the molecular basis for differences in the biological activities of the two compounds.

The metabolites of 6-MBaP formed from the metabolism of this compound by liver microsomes prepared from male Sprague-Dawley rats pre-treated with either of two agents that induce particular forms of cyto-chrome P-450, 3-methylcholanthrene and phenobarbital, or from liver enzymes from animals who received no pretreatment, have been isolated by reversed-phase and normal-phase high performance liquid chromatography and have been identified by ultraviolet-visible absorption spectroscopy and mass spectroscopy. The amount of each 6-MBaP metabolite formed

depended on the microsomal preparation used.

Like BaP, 6-MBaP is metabolized to 4,5-, 7,8-, and 9,10-dihydrodiols (diols) and several monohydroxylated metabolites of the parent hydrocarbon including two not found to have BaP metabolite counterparts. Unlike BaP, 6-MBaP is not metabolized to quinones; however, metabolism at the six-methyl group results in the formation of 6-hydroxymethylbenzo[a]pyrene (6-OHMBaP) which can be further metabolized to both the 1- and 3-phenols of 6-OHMBaP.

Quantitative comparisons of the microsomal metabolism of [^{3}H]-6-MBaP and [^{14}C]BaP revealed decreases of 72 to 96% in the rates of formation of the 6-MBaP 4,5-, 7,8-, and 9,10-diols compared with those of the BaP diols and decreases of 66 to 91% in the overall rates of formation of 6-MBaP metabolites relative to those of BaP metabolites, depending on the type of microsomes used.

Nuclear magnetic resonance and circular dichroism spectrophotometric characterization of the 6-MBaP diol metabolites formed from the microsomal metabolism of 6-MBaP showed that like BaP, 6-MBaP is stereoselectively metabolized to trans-4,5-, 7,8-, and 9,10-diols in which the [R,R] enantiomer predominates. While the BaP 4,5- and 7,8-diols preferentially adopt the quasi-diequatorial conformation, steric hindrance by the six-methyl group of 6-MBaP forces the hydroxyls of the corresponding 6-MBaP diols to preferentially adopt quasi-diaxial conformations. The hydroxyls of both the 6-MBaP and BaP 9,10-diols favor the quasi-diaxial conformation because they lie in bay regions.

Based on the results of this study, numerous steps in the metabolic pathways involved in the in vitro microsomal metabolism of 6-MBaP have been established. The relatively lower rates of formation

of the 6-MBaP 7,8-diol may account, in part, for the decreased carcinogenic activity of 6-MBaP compared to that of BaP since relatively less 6-MBaP 7,8-diol would be available for further metabolism to diol epoxides. However, the relative biological activities of 6-MBaP and BaP may also depend on other factors such as the different conformational preferences of their respective trans-7,8-diol metabolites.

A COMPARATIVE STUDY OF THE METABOLISM OF 6-METHYLBENZO[a]PYRENE AND BENZO[a]PYRENE BY RAT LIVER MICROSOMES

by

Karen Lee Hamernik

Dissertation submitted to the Faculty of the Department of Pharmacology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1984

DEDICATION

To my parents, Mildred and Daniel-

with love and appreciation.

ACKNOWLEDGEMENT

Special thanks to Mr. Henry Weems, Department of Pharmacology, USUHS, for his assistance in obtaining the mass spectral data and to Dr. Peter Fu, National Center for Toxicological Research, Jefferson, Arkansas, for his assistance in obtaining and interpreting the NMR spectral data.

I am especially indebted to Henri Weems, Pei-Lu Chiu, Spencer Ueng, Bob Whitmore, Ronnie and Linda Bryant, Debra Callicutt, Kathy Reed, and Mark and Sue Lindeman for their help and constant encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

BACKGROUND

Introduction

Over five decades of investigation have been devoted to the study of polycyclic aromatic hydrocarbons (PAH). While these compounds can form as a result of natural phenomena (1), large quantities resulting from industrial processes, automobile emissions, open burning, and power generation are released as particulate matter into the environment (2). In addition, PAH are present in cigarette smoke and charcoal broiled meats (3,4). Questions concerning the effects of this chemical burden on human health, and plant and animal life have prompted extensive research efforts on the part of both the scientific and medical communities. Since the onset of such studies, PAH have been shown time and again to be carcinogens and mutagens in mammalian systems and there is evidence to indicate that these compounds may play an important role in the etiology of human cancer (5-8). Human exposure to PAH is possible through various routes which include inhalation, ingestion of food and water, and absorption through the skin (9).

Metabolism of PAH

In order for PAH and other fat soluble xenobiotics (drugs, pesticides, etc.) to be eliminated from the body, they must first be converted to more water soluble derivatives. Most of this biotransformation occurs in the liver but significant extrahepatic metabolism, particularly in the tissues of the lung, skin, intestine, and kidney is known to occur (9,10).

The principal enzyme systems involved in xenobiotic biotransformation function physiologically to metabolize endogenous substrates such

as hormones, fatty acids, and cholesterol and are located in the cellular endoplasmic reticulum (10).

In vitro metabolism studies are often carried out using preparations of the liver endoplasmic reticulum referred to as microsomal enzyme systems (microsomes) (11,12). Three protein components of these enzyme systems important in the metabolism of PAH are the heme-containing cytochrome P-450 monoxygenases (mixed function oxidases), NADPH-cytochrome P-450 reductase, and epoxide hydrolase (EH). The net result of cytochrome P-450 and the reductase working in concert is the insertion of one atom of atmospheric oxygen into the PAH substrate. NADPH is a necessary mediator in the reaction sequence in that it provides at least one of the two electrons essential to the oxidative process. This electron is transferred in turn to the cytochrome via the reductase. The origin of the second electron and the mechanism by which it is transferred into the cytochrome-oxygen-substrate complex is still uncertain (11,13,14). EH is located in the endoplasmic reticulum of many tissues, including the liver, in close association with the cytochrome P-450's (21). This enzyme catalyzes the addition of water to arene oxides to form dihydrodiols (21).

Microsomal Metabolism of Benzo[a]pyrene

When a PAH such as the widely studied compound benzo[a]pyrene (BaP) is metabolized in vitro by rat liver microsomes, a number of unconjugated primary, secondary, and tertiary metabolites are formed and many have been isolated by high performance liquid chromatography (HPLC) (for review see 15).

The initial step in the metabolism of BaP is an epoxidation reaction catalyzed by the mixed function oxidases (MFO) which yields any one

of five possible arene oxides (1,2-, 2,3-, 4,5-, 7,8-, and 9,10-oxides) (8,16-18). These oxygenated derivatives can either rearrange to form phenols (19) via an NIH shift mechanism (20) or instead can be hydrated by EH to form dihydrodiols (diols) (21). Three diols ((-)-trans-4,5-, (-)-trans-7,8-, and (-)-trans-9,10-diols) (17,22,23) and a number of phenols (1-, 3-, 7-, and 9-phenols) (17,24,25) have been identified. Mechanistic studies which have been conducted cannot eliminate direct enzymatic hydroxylation as a possible means of BaP phenol formation (16). A certain amount of the 9- and 7-phenols may arise nonenzymatically (22,26).

The BaP 6-phenol, a short-lived compound never isolated by HPLC, is purported to be a major primary oxidative metabolite of BaP (27). Although the exact mechanism of 6-hydroxylation is uncertain (28), it is thought to proceed enzymatically since liver homogenates fortified with NADPH are required for the formation of 6-hydroxy BaP which has been detected by electron spin resonance spectrophotometry (ESR) as a 6-oxy free radical (29-31).

Once formed, 6-OH BaP is thought to be autooxidized via a 6-oxy free radical intermediate to three quinone metabolites of BaP (1,6-, 3,6- and 6,12-quinones) (17,30,32,33). Quinones may also arise via a cytochrome P-450-mediated one electron oxidation of BaP to a radical cation which, in turn, could be oxidized, via a cytochrome P-450-mediated transfer of oxygen, to the 6-oxy free radical intermediate (34). In addition, some 3,6-quinone is formed by further chemical or enzymatic oxidation of the BaP 3-phenol (16,35,36).

The BaP 7,8-diol can be further metabolized by the MFO to the 7,8-dihydrodiol 9,10-epoxide (diol epoxide) metabolite (37,38). Micro-

somal metabolism of the (+) and (-) enantiomers of the BaP 7,8-diol yielded four stereoisomeric 7,8-diol 9,10-epoxides which were isolated by HPLC as tetrols and triols (39).

The formation of other BaP metabolites such as the BaP 9-phenol 4,5-oxide is reviewed elsewhere (28).

Oxygenated metabolites of BaP can be conjugated to form glutathione, glucuronide, or sulfate derivatives by transferases which are present in the liver endoplasmic reticulum and cytosol as well as in other tissues (10,40). These conjugates can form in vitro in the presence of the soluble fraction of liver cell homogenates and the appropriate cofactors (41-43).

Activation as well as detoxification can occur when PAH are metabolized (44). Although some metabolites of BaP have little or no toxicity in biological systems, others, such as arene oxides and diol epoxides, can covalently bind to cellular macromolecules such as DNA, RNA, and protein (45-47). These interactions can result in the initiation of mutation or cancer. Studies have shown that BaP, as well as other PAH, must be metabolically activated to exert their toxic effects (48,49). Generally, a detoxification function has been attributed to conjugation reactions. However, some conjugates can be cleaved enzymatically or non-enzymatically to yield reactive species or their precursors (50). Examples include the sulfate esters of N-hydroxy-2-acetylaminofluorene and 6-hydroxymethylbenzo[a]pyrene (6-OHMBaP) (51,52).

Inducers of Microsomal Enzyme Activity

The activity of the enzymes which metabolize PAH is affected by the species, strain, age, sex, and nutritional status of the animals used and the particular tissues from which the enzymes are derived.

Many exogenous compounds can act as inducers, inhibitors and/or stimulators of microsomal enzyme activity *in vivo* and *in vitro*. At present there are six categories of chemical agents which have selective effects on MFO and on EH and conjugase enzyme activity. Such compounds can actually modulate the detoxification and activation pathways of PAH metabolism, can shift metabolic paths in favor of particular metabolites, and therefore, have the potential to interfere with or enhance the induction of mutagenicity or carcinogenicity (53; for reviews see 10,54,55).

3-Methylcholanthrene (3-MC) (a PAH) and phenobarbital (PB) (a drug) are two of the best studied inducing agents for which categories have been named (56,57).

Cytochrome P-450 Induction

Multiple forms of cytochrome P-450 have been isolated and purified from different tissues of different animal species. There are at least six distinct forms of the cytochrome present in rat liver microsomes which differ in their spectral properties, catalytic activity towards a number of substrates, electrophoretic mobilities, immunological properties, and amino acid sequence (for reviews see 10,60-62). Induction by PB or 3-MC is distinguished by the ability of each of these agents to elevate levels of specific cytochrome P-450's (63). Early studies showed that the *de novo* synthesis of the cytochrome selectively induced by 3-MC was prevented by inhibitors of protein and RNA synthesis (48,64). More recent studies have shown that the formation of specific mRNA's which code for the synthesis of specific cytochrome P450's is stimulated by the administration of PB to rats (65,66). A cytosolic receptor has been implicated in the mechanism of 3-MC induction (67).

Effects of Inducers on Spectral Properties

When the cytochrome P-450 content of hepatic microsomes prepared from rats which had been pretreated with 3-MC or PB was determined, it was found that PB preferentially induced cytochrome P-450_b with a carbon monoxide-induced difference spectral peak at 450 nm, while 3-MC induced predominantly cytochrome P-450_c with spectral peak at 448 nm (63, 171). Unlike 3-MC, PB-pretreatment causes a proliferation of rat liver smooth endoplasmic reticulum, increases the total amount of microsomal protein, and increases total liver weight (68). The cytochrome P-450 present in microsomes prepared from untreated rats has spectral properties similar to those of the PB-inducible form (171).

Effects of Inducers on Enzyme Catalysis

The inducible forms of cytochrome P-450 have characteristic albeit overlapping substrate specificities and show differences in the rates at which these substrates are metabolized.

In rats, 3-MC preferentially induces cytochrome P-450_c-catalyzed aryl hydrocarbon hydroxylase (AHH) activity* (63,171). Although it does not increase the catalytic activity of ethylmorphine-N-demethylase or NADPH-cytochrome P-450(c) reductase above control levels (171), it potentiates 7-ethoxycoumarin-O-dealkylation (63) and has been found to be a weak and variable inducer of EH activity (73).

Cytochrome P-450_b-catalyzed AHH activity is selectively induced by PB (63,171). PB-pretreatment more than doubles ethylmorphine-N-demethylase and NADPH-cytochrome P-450(c) reductase activities above control levels (171), only slightly increases 7-ethoxycoumarin-O-

*The AHH assay measures the metabolic conversion of BaP to primarily the highly fluorescent metabolite 3-OH BaP.

dealkylase activity (63), but is an excellent inducer of EH activity (73). Differences in the rate and extent of BaP metabolism by 3-MC-, PB-, and control microsomes and by their respective purified cytochrome P-450's have been reported (17,69).

Effects of Inducers on the Regio- and Stereoselectivity of PAH Metabolism

When a particular PAH undergoes metabolism by microsomal enzymes, there are certain regions of the molecule which are more likely to be attacked than others (70,71). This regioselectivity of MFO can be altered by chemical agents which preferentially induce or inhibit particular forms of cytochrome P-450 (59,69). As a result, alterations can occur in the amounts of metabolites produced when a PAH substrate such as BaP is metabolized by rat liver enzymes derived from animals which have been pretreated with agents such as 3-MC or PB, or which received no pretreatment (17). (For examples and other references see next section-"Regio- and Stereoselectivity in the Metabolism of Benzo[a]pyrene").

Studies examining the metabolism of BaP to mutagenic metabolites have shown that both cytochrome P-450 and EH play critical roles in the activation/detoxification pathways of PAH metabolism (for review see 21). In addition, 3-MC and PB can affect PAH metabolism by changing the mixed function oxidase/epoxide hydrolase ratios (72). Shifts in the balance of these enzyme activities are possible because both PB and 3-MC are inducers of MFO, while only PB shows significant ability to induce EH activity (73). Since glucuronide conjugation reactions can be enhanced by either PB or 3-MC depending on the substrate involved (74), induction of these conjugation enzymes might also have an important

effect on PAH detoxification and activation.

Mammalian microsomal enzyme systems have been found to preferentially or exclusively attack a particular face of the substrate PAH undergoing metabolism (59,71). The final geometry (absolute configuration) and stereoisomeric constituency of PAH diol and diol epoxide metabolites is largely dependent on this stereoselective or stereospecific attack (39,75,76). Distinct differences in stereoselectivity in the metabolism of PAH have been observed with untreated, PB-, and 3-MC-pretreated liver enzymes (39). (For examples and other references see next section "Regio- and Stereoselectivity in the Metabolism of Benzo[a]pyrene"). The effects of exogenous chemicals such as PB and 3-MC on this aspect of metabolism is of critical importance since the carcinogenicity and mutagenicity of PAH in a particular living system has been shown to be intrinsically related to the stereochemistry as well as amounts and potencies of the active metabolite(s) (77-80 and discussion in 81).

Regio- and Stereoselectivity in the Metabolism of Benzo[a]pyrene

Regioselective differences in the metabolism of BaP by rat and rabbit liver enzymes pretreated with 3-MC or PB, or by reconstituted enzyme systems containing purified induced forms of cytochrome P-450 are reflected quantitatively in the profiles of metabolites formed (17, 26,69-71,82). For example, pretreatment with 3-MC favors formation of the 9,10- and 7,8-diols, whereas, pretreatment with PB favors formation of the 4,5-diol (17,26). In addition, the ratio of 9-OH BaP to 3-OH BaP formed from the metabolism of BaP was found to vary with 3-MC- or PB-pretreatment (69).

Induction by 3-MC greatly enhances the percent of substrate meta-

bolized as well as the catalytic rate of conversion of BaP to both fluorescent and total metabolites compared with PB induction or no pretreatment (17,69).

Purified cytochrome P-448 more effectively converted BaP to mutagenic metabolites than did cytochrome P-450. The higher specific activity of cytochrome P-448 compared to cytochrome P-450 in this conversion indicates differences in the active site of the two hemoproteins (83). At least one model for the catalytic site of cytochrome P-448 has been proposed (84).

An important advance in the understanding of the biological activities of BaP and other PAH came with the discovery that MFO had various degrees of stereoselectivity in the metabolism of these compounds to reactive intermediates (for reviews see 28,39,54,55,78,85).

Liver microsomes from rats pretreated with 3-MC stereospecifically converted BaP to only one of the two possible 7,8-diol enantiomers, the potently carcinogenic and mutagenic (-)-trans-7,8-diol (37) (see Fig. 1). The mechanism of formation of this isomer involves the MFO-catalyzed attack of molecular oxygen on the upper face of the BaP molecule to yield exclusively the BaP (+)-[7R,8S]-oxide. EH then stereospecifically hydrates the arene oxide by cleavage at the carbon 8 position to form the (-)-trans-[7R,8R]-diol (22,39). This diol is converted stereoselectively by MFO to predominantly the BaP (+)-trans-7,8-diol 9,10-epoxide, the metabolite postulated to be the ultimate carcinogen of BaP (75). When microsomes from PB-treated or control rats were used, metabolism was less stereospecific in either the conversion of BaP to the 7,8-diol or the subsequent conversion of the diol to diol epoxides such that certain percentages of other possible,

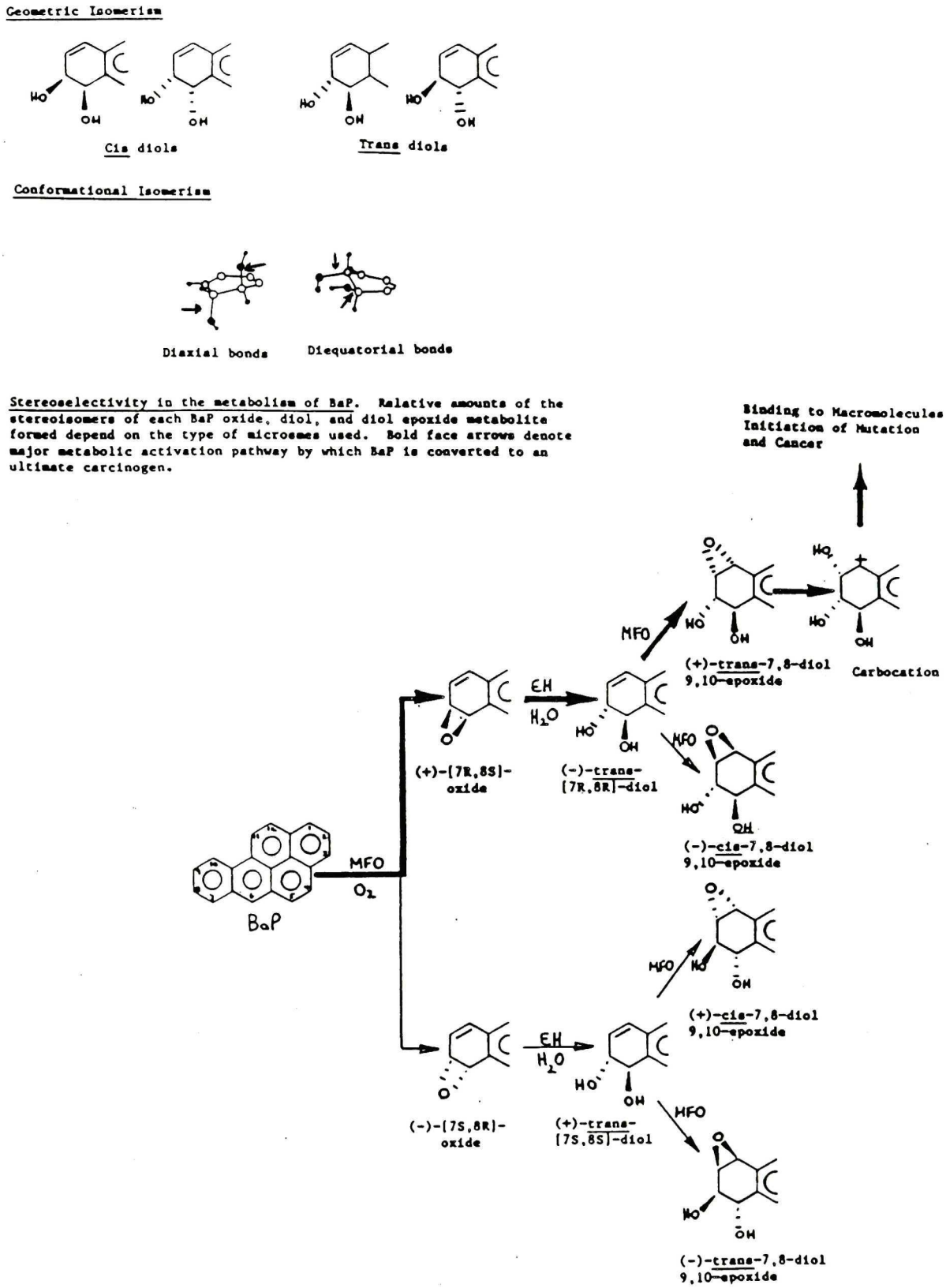


Fig. 1. Isomerism and Stereoselectivity in the Metabolism of BaP. Triangle bonds are oriented towards the viewer; dashed bonds are oriented away from the viewer.

less tumorigenic isomers were formed (39,78).

Thus chemical agents can dramatically affect the pathways that lead to reactive chemical species in BaP metabolism and the amounts of these active metabolites which form.

Mutagenicity, Carcinogenicity, and Covalent Binding of BaP and Derivatives

The theory that BaP is biologically activated via a pathway that proceeds consecutively from the parent hydrocarbon through two proximate carcinogen intermediates, the BaP (+)-7,8-oxide and (-)-trans-7,8-diol to the ultimately carcinogenic 7,8-diol 9,10-epoxide has been supported by evidence obtained from mutagenicity, carcinogenicity, and covalent binding studies (86, and see reviews in 28,54,55,87).

BaP and most of its known and potential primary and secondary metabolites (quinones, diols, phenols, oxides) were not found to possess substantial intrinsic mutagenic activity (with the exception of BaP 4,5-oxide and BaP 6-OH) in the Ames Salmonella typhimurium mutagenicity assay or Chinese hamster V79 cell testing system (88). However, when a PAH metabolic activating system was added to either the bacterial or mammalian cell bioassay, the most extensive conversion to mutagenic metabolites of any of the compounds tested was shown by the BaP trans-7,8-diol followed by that of BaP and BaP 7,8-oxide. Other oxides, diols, quinones, and phenols were inactive or were less actively converted to mutagens than was BaP (89,90).

Further reinforcement of the notion that the BaP 7,8-diol 9,10-epoxide was the ultimate carcinogen of BaP came from mutagenicity studies performed with S. typhimurium strains TA 98 and TA 100 and Chinese hamster V79 cells. These experiments demonstrated the high

inherent mutagenic activities of the racemic cis and trans BaP 7,8-diol 9,10-epoxides* (91,92) relative to those of BaP and fifteen of its oxide, phenol, diol, quinone, and other diol epoxide derivatives in either the bacterial or mammalian cell bioassay. When the optically pure (+) and (-) enantiomers of each racemate were purified and their mutagenic activities determined in both testing systems, the (-) enantiomer of the cis BaP 7,8-diol 9,10-epoxide was up to 9.5 times more mutagenic than were the other three stereoisomers in the bacterial system. However, in V79 cells, the (+) enantiomer of the trans BaP 7,8-diol 9,10-epoxide was up to 18 times more mutagenic compared to the other three stereoisomers (80). The exact reason for the differences seen in the two systems is still speculative.

Only carcinogenicity studies can verify a metabolite or compound as a proximate or an ultimate carcinogen. The results of several types of carcinogenicity studies, wherein the compound to be tested was applied to mouse skin as a complete carcinogen or in a two-stage initiator-promotor regimen with phorbol ester, or was injected subcutaneously into newborn mice, demonstrated the high tumorigenic potencies of the BaP trans-7,8-diol (93-96), and the trans BaP 7,8-diol 9,10-epoxide (97) compared to BaP or other BaP derivatives. The BaP 7,8-oxide was also active as a tumorigen (94,98). Neither the BaP 6-phenol (99) nor the 4,5-oxide (98) showed significant tumorigenic activity. The BaP 2-

*The cis BaP 7,8-diol 9,10-epoxide is composed of two enantiomers each with relative configuration such that the 9,10-epoxide oxygen is cis to the 7-benzylic hydroxyl group. This stereoisomer is also known as the syn isomer, diol epoxide II, or diol epoxide 1. The trans BaP 7,8-diol 9,10-epoxide is composed of two enantiomers each with relative configuration such that the 9,10-epoxide oxygen is trans to the 7-benzylic hydroxyl group. This stereoisomer is also known as the anti isomer, diol epoxide I, or diol epoxide 2.

phenol was quite active; however, this compound has not at present been shown to be a metabolite of BaP (100). Interestingly, the BaP H_4-7,8-epoxide and BaP H_4-7,8-diol, two compounds whose 9,10-double bonds are saturated, lack carcinogenicity implying that the carcinogenicity of BaP 7,8-oxide and BaP 7,8-diol was due to the metabolic activation of these compounds to 7,8-diol 9,10-epoxides (93,94).

Further studies with the optically pure (+) and (-) enantiomers of BaP 7,8-oxide and trans-7,8-diol showed, that in each case, the tumorigenicity of one particular isomer was far greater than for the other (101-103). The isomers with the higher activities were the (+)-7,8-oxide and the (-)-trans-7,8-diol. The (+) enantiomer of the trans BaP 7,8-diol 9,10-epoxide was the only one of the four possible diol epoxide stereoisomers to show significant tumorigenicity (79,104).

When BaP was applied topically to the backs of mice, the (+) enantiomer of the trans BaP 7,8-diol 9,10-epoxide was found to be the major adduct covalently bound to DNA, RNA, and protein of mouse skin. Most of the binding of this isomer to DNA and RNA occurred at the exocyclic 2-amino group of guanine as it did in vitro to double-stranded DNA (105-107).

Bay Region Theory of Carcinogencity

Currently the most widely accepted hypothesis correlating PAH structure and activity is the bay region theory. Jerina and colleagues postulated that PAH bay region vicinal diol epoxides should be the metabolites with the highest chemical and biological activity since the common structural feature of several PAH with highly reactive diol epoxides was the presence of the epoxide located in a bay region and situated on an angular benzo ring (81,86,108). An additional basis for

their theory came from DNA binding studies by Borgan et al. (109) and Sims et al. (110) which implicated the BaP vicinal 7,8-diol 9,10-epoxide as the ultimate carcinogen of BaP.

The binding of the vicinal bay region diol epoxide to DNA is believed to occur by the solvolytic opening of the benzylic oxirane carbon-oxygen bond to form an electrophilic carbonium ion (111). Dewar's pertubational molecular orbital calculations of the ease of benzylic carbocation formation (as given by $\Delta\varepsilon_{deloc}/\beta$)* was used in support of the bay region theory to predict the relative chemical activities of many PAH diol epoxides (112). It was assumed that the more readily the electrophilic carbonium ion formed, the higher the $\Delta\varepsilon_{deloc}/\beta$ and the greater the tendency for electrophilic attack on the DNA molecule. In many cases this quantum mechanical model of chemical reactivity readily predicted the relative biological activities of a series of unsubstituted PAH which had different carcinogenic or mutagenic potencies (81, 86,108,111,113). However, this model was shown to be not totally effective in predicting PAH carcinogenicity. For example, benzo[e]-pyrene was not found to be a tumorigenic compound although its $\Delta\varepsilon_{deloc}/\beta$ predicted that its activity should be substantial (111,114). In fact, the bay region theory does not take into account the relative abilities of the cytochrome P-450's and EH to convert parent PAH to their respective diols or to convert these diols to diol epoxides, nor does it take into account the effects of substituents or stereochemical factors (relative configuration, absolute configuration, and conformation) in its predictions (81).

*The π-electron energy change in terms of a parameter, $\Delta\varepsilon_{deloc}$, in units of the resonance integral, β.

Other Electrophilic Species

Based on evidence from a number of studies, mechanisms of PAH activation to electrophilic reactants other than the bay region vicinal diol epoxide have been postulated although their relative contribution to the total biological activity of BaP and other PAH is unknown (115, 116, and for reviews see 34 and 117).

One of these alternant electrophiles is a 6-oxy free radical generated at position six of BaP, the most chemically reactive site on the molecule (29-31,118, and see discussion in previous section-"Microsomal Metabolism of Benzo[a]pyrene"). Another reactive species of BaP is a free radical cation (117,119) which can be produced via a one electron oxidation of BaP (119-121).

Evidence for the nucleophilic trapping of the BaP radical cation, in vitro by DNA isolated from rat liver nuclei and in vivo by mouse skin DNA, was obtained from studies which determined the tritium loss from specific positions on the BaP molecule after electrophile-nucleophile reaction had taken place. Binding was shown to occur predominantly at the six-position of the BaP molecule, the region of highest charge density (122). The 6-oxy free radical has also been found as a DNA adduct and has been detected by ESR in rat liver nuclei (29,123).

Rationale for Study of 6-Methylbenzo[a]pyrene Metabolism

Addition of a methyl substituent at any of the twelve possible substitution sites on the BaP molecule has been shown to enhance or diminish the carcinogenicity of this compound (7,124-127). Investigations designed to determine the biological basis for these differences has just begun since they cannot readily be accounted for by the original postulates of the bay region theory (81).

6-Methylbenzo[a]pyrene (6-MBaP) was chosen for study in the investigations to be elaborated here for a number of reasons:

In a two-stage mouse skin tumorigenicity testing system and in several other types of carcinogenicity studies, 6-MBaP was found to be moderately active as a tumorigen although with potency less than that of BaP (96,127-129). In contrast, comparative mutagenicity studies of 6-MBaP and BaP showed 6-MBaP to be the more active compound (52,130, 131). It had not been determined how the presence of the six-methyl group resulted in differences in the relative biological activties of BaP and 6-MBaP. Although the primary activation pathway of BaP had been shown to proceed via a (-)-trans-7,8-diol intermediate to the ultimately reactive (+)-trans-7,8-diol 9,10-epoxide, a similar pathway in the metabolism of 6-MBaP had not been established. The 6-methyl group of 6-MBaP is located in a position peri (adjacent) to the terminal benzo ring. In formulating the bay region theory, it was noted by Jerina and colleagues that the presence of an alkyl (i.e. methyl) or halogen (i.e. fluoro) substituent located in a position peri to a terminal benzo ring which formed part of a bay region had the effect of decreasing the tumorigenicity of the substituted PAH relative to that of the PAH without the substituent (19,81). In context of the bay region theory, the molecular basis for this peri effect could involve one or more factors: A peri alkyl or halogen substituent could reduce metabolism at the adjacent terminal ring positions (positions 7 and 8 of 6-MBaP). The regioselectivity of the cytochrome P-450's towards the metabolism of BaP in the formation of the BaP 7,8-oxide and the efficiency of EH towards the hydrolysis of this epoxide could be altered by the presence of a 6-methyl group. However, additional factors must also be con-

sidered. Due to steric crowding and/or electronic repulsion (depending on the substituent), conformational transformations would be induced in a dihydrodiol metabolite which might form at positions of the terminal benzo ring located peri to an alkyl or halogen substituent (i.e. the 7,8-dihyrodiol metabolite of 6-MBaP). 6-MBaP is the only monomethylated isomer of BaP whose trans-7,8-diol metabolite would preferentially adopt a conformation different from that of the BaP trans-7,8-diol metabolite. Conformational alterations in a key dihydrodiol metabolite could result in decreases in its further metabolism to bay region vicinal diol epoxides and/or could effect the relative reactivity of a bay region vicinal diol epoxide metabolite which did form.

Two possible conformations have thus far been reported for PAH trans diols formed by mammalian metabolic enzyme systems. In the quasi-diequatorial (EE) conformation, the diol hydroxyls lie in the plane of the molecule and this conformation has been reported for the hydroxyl groups of the BaP (-)-trans-7,8-diol and the BaP (-)-trans-4,5-diol. Alternately, hydroxyl groups can lie above and below the plane of the molecule in the quasi-diaxial (AA) conformation. This conformation is preferentially adopted by the hydroxyl groups of the BaP (-)-trans-9,10-diol and is the conformation anticipated for a diol metabolite which forms in a baylike region brought about by a peri methyl substituent (see references 133-139 and discussions of PAH diol conformation therein).

The biological activity of BaP is also dependent, in part, on its stereoselective conversion by metabolic enzyme systems to particular stereoisomers of the 7,8-diol and 7,8-diol 9,10-epoxide. The effects of the six-methyl group on the conversion of 6-MBaP to optically active

metabolites is not known.

Additional interest in 6-MBaP was sparked from studies which proposed that metabolites other than the 7,8-diol and bay region vicinal 7,8-diol 9,10-epoxide might play a role in the activation of this compound.

Cavalieri and colleagues have suggested that a one electron oxidation of 6-MBaP to a free radical cation might be an important method of bioactivation for this compound (34,116,117). His hypothesis is based on the high ionization potential of 6-MBaP which is a reflection of the compound's ability to form a radical cation; on the results of studies examining the binding of 6-MBaP to DNA in vitro in a horseradish peroxidase/hydrogen peroxide-catalyzed reaction system; and on the results of studies examining the binding of 6-MBaP to mouse skin DNA in vivo. 6-MBaP was reported to bind to the nucleophile at the 6-methyl group although solid evidence to support this conclusion was not given (58,117, and reviewed in 34). In a discussion of these experiments, it was suggested that differences in the abilities of 6-MBaP and BaP to form radical cations might account, at least in part, for differences in their relative carcinogenicities.

Another postulated mechanism of activation of 6-MBaP involves the initial biotransformation of this compound to 6-OHMBaP (141). Most studies comparing the carcinogenic activity of 6-OHMBaP and 6-MBaP showed the parent compound to be more active than the derivative depending on the tumor model system used (96,116,128,129,141,142). However, further studies showed that 6-OHMBaP might first have to be converted enzymatically to a reactive ester with a good leaving group which could in turn form a benzylic carbonium ion capable of covalently binding to

DNA. Binding appeared to be ATP-dependent (52,115,129,140,143). The sulfate ester of 6-OHMBaP has been found to have both significant carcinogenic and mutagenic activity and is purported to form *in vitro* from the metabolic activation of 6-OHMBaP by a rat liver cytosolic sulfotransferase-PAPS generating system in the presence of ATP (52,116,128, 170, and reviewed in 34). Sulfate ester formation is thought to play a role in the bioactivation of 7,12-dimethylbenz[a]anthracene and 2-acetylaminofluorene to mutagens and has been implicated in the mechanism of carcinogenicity of these two compounds (51,52,144).

The controversy surrounding 6-MBaP as a biologically active compound necessitated a better understanding of the metabolic pathways of this PAH. Since reliable qualitative and quantitative data on the metabolism of 6-MBaP were lacking, the current study was undertaken.

In broad perspective, these investigations were carried out to determine the effect of methyl substitution at position six on the metabolism of 6-MBaP relative to that of BaP. It was felt that this was a necessary first step to the eventual understanding of the molecular basis for the differences in the biological activities of these two compounds which differ only in the methyl substituent.

Practically, there were four aspects to the study:

The first aspect was qualitative in nature. The metabolites of 6-MBaP formed *in vitro* by rat liver microsomes were isolated and identified to determine if there were differences or similarities in the metabolic profiles of 6-MBaP and BaP. A determination of the effects of the *peri* methyl substituent on the conformation and geometric configurations of the 6-MBaP diol metabolites was also made.

In the second aspect of the investigations, a quantitative study

was made of the relative amounts of 6-MBaP and BaP metabolites formed by rat liver microsomes prepared from rats pretreated with 3-MC or PB, or from animals which received no pretreatment (control microsomes). This was done for the following reasons: to compare the effects of the six-methyl group on total metabolism of the two substrates; to see if the peri methyl group directed metabolism away from the terminal benzo ring to other sites on the molecule; to see if there were differences in the major metabolites of BaP and 6-MBaP; to compare the relative amounts of 6-MBaP and BaP 7,8-diol formation and that of the other diol metabolites of BaP and 6-MBaP; to determine any differences in the regioselective metabolism of the two substrates which might be brought about by prior treatment of the liver enzymes with the inducing agents 3-MC and PB, and to compare the results obtained using the induced preparations with those obtained using the control preparation.

In the third aspect of the study, the comparative metabolism of 6-MBaP and BaP to optically active diol metabolites was investigated.

Finally, qualitative and quantitative investigations of the further metabolism of 6-OHMBaP were undertaken because several 6-OHMBaP phenol metabolites were found to be metabolites of 6-MBaP.

MATERIALS AND METHODS

Materials

Substrates. BaP and specifically labelled [7,10-^{14}C]BaP (26 mCi/mmol) were purchased from Aldrich Chemical Co., Milwaukee, Wisc. and Amersham Radiochemicals, Arlington Heights, IL., respectively. Unlabelled 6-MBaP and 6-OHMBaP were synthesized according to established procedures (145-147) by Dr. Peter P. Fu (National Center for Toxicological Research, Jefferson, Arkansas). Synthesis of generally labelled [^{3}H]6-MBaP (62 mCi/mmol) by catalytic exchange in solution with tritium gas and of specifically labelled [6-CH_2-^{3}H]6-OHMBaP (141 mCi/mmol) by reduction of BaP 6-carbaldehyde with sodium borotriteride, were also performed by Amersham Radiochemicals.

Due to the presence of impurities, it was necessary to initially purify labelled or unlabelled substrate by passage of the compounds over a silica gel column (Bio-Sil A, 100-200 mesh, Bio-Rad Labs, Richmond, Ca.) with 100% hexane (labelled and unlabelled BaP and 6-MBAP) or 25% ethyl acetate in hexane (labelled and unlabelled 6-OHMBaP) as the eluting solvents. Final purification steps were carried out as required by reversed- and normal-phase HPLC. The final purity of radiolabelled substrates was established by HPLC-fractionation techniques identical to those used in the quantitation experiments. [^{14}C]BaP and [^{3}H]6-MBaP were found to have purities of greater than 99%. [^{3}H]6-OHMBaP was 98% pure. Before use, labelled substrates were diluted to the indicated specific activities with unlabelled substrate and diluted to appropriate concentrations with organic solvent. Identities of substrates were confirmed by mass spectroscopy (non-labelled) or by elution of labelled

substrate with authentic unlabelled standards.

The extinction coefficient of 6-OHMBaP (56,823 cm^{-1} M^{-1} at a wavelength maximum of 301 nm) was determined spectrophotometrically with known preweighed amounts of purified compound dissolved in methanol.

Animals. Male 80 to 100 g Sprague-Dawley rats (Taconic Farms, Germantown, N.Y.) were used for preparation of microsomal enzymes. Groups of four animals were housed in plastic cages with metal screen tops under a controlled light/dark cycle on wood-chip bedding. The rats were allowed free access to food (Agway Rat Chow) and water.

Other. Synthetic BaP metabolite standards were purchased from the Chemical Respository of the National Cancer Institute, Bethesda, MD., while [^{3}H]H_2O and [^{14}C]toluene liquid scintillation calibration standards came from Amersham Radiochemicals. The liquid scintillation cocktail used in the quantitative studies (Redi-Solv HP) was purchased from Beckman, Fullerton, CA. All other reagents, solvents, biochemicals, and materials were purchased from commercial sources.

Methods

Microsome preparation. Selective induction of liver microsomal enzyme activity was achieved by the intraperitoneal injections of animals with sodium phenobarbital (Mallinckrodt, St. Louis, MO.) dissolved in water (75 mg/kg/day for three days) or 3-methylcholanthrene (Sigma Chemical Co., St. Louis, MO.) dissolved in corn oil (25 mg/kg/day for four days) in standard dosage regimens. Control enzyme preparations were made from animals which received no pretreatments. Hereafter, the three microsomal preparations will sometimes be referred to as

3-MC-, PB-, or control microsomes. As a result of pretreatment, the cytochrome P-450 contents of 3-MC- and PB-microsomes were induced to extents comparable with those obtained by Alvares et al. (171).

Animals were sacrificed by cervical dislocation 24 hours after the last injection of inducer. Livers were promptly removed and placed in 0.25 M sucrose-0.05 M TRIS-HCl buffer, pH 7.5. Microsomes were prepared from fresh liver homogenates as described in (148) with several modifications. Briefly, liver homogenates were centrifuged for 15 min at 9000 x g. The supernatant was then centrifuged at 105,000 x g for 1 hr to obtain the microsomal pellet. Microsomes equivalent to 1 gm liver, wet weight, were resuspended in buffer and stored as suspensions at -80°F prior to use. The microsomal protein concentrations and cytochrome P-450 contents were obtained as described respectively by the methods of Lowry (149) and Omura et al. (150). A Carey model 118 spectrophotometer, (Varian Instruments, Palo Alto, CA.) and an Aminco DW-2a spectrophotometer, (American Instrument Co., Silver Spring, MD.) were used respectively in making these determinations. The AHH activity of each preparation was determined fluorimetrically, using a Model MPF-44A fluorescence spectrophotometer, (Perkin-Elmer Corp., Norwalk, Conn.) essentially by the method of Nebert et al. (151) with modifications as described in (152). For the comparative quantitative studies of [^{3}H]6-MBaP and [^{14}C]BaP metabolism, the cytochrome P-450 contents (nmol cytochrome P-450 per mg protein) of 3-MC-, PB-, and control microsomes were determined to be 0.99, 1.81, and 0.49 respectively. For the same studies, the AHH activity (pmol 3-OH BaP per mg protein per minute) for 3-MC-, PB-, and control microsomes were respectively 1500.3, 229.7, and 76.2. In a separate series of experi-

ments in which the conversion of [^{3}H]6-OHMBaP to its metabolites was studied, the cytochrome P-450 contents of 3-MC-, PB-, and control microsomes were 1.32, 1.48, and 0.38 nmol cytochrome P-450 per mg protein respectively. AHH activity of these preparations was not determined.

In vitro incubations. For qualitative studies, metabolites of 6-MBaP, 6-OHMBaP, or BaP were obtained from large volume incubations (> 100 ml) of substrate with 3-MC-, PB-, or control microsomes. Each ml of incubation mixture (pH 7.5) contained: 50 µmol TRIS-HCl, 3 µmol $MgCl_2$, 2 µmol glucose-6-phosphate, 0.1 unit of glucose-6-phosphate dehydrogenase (Type II, Sigma Chemical Co., St. Louis, MO.), 96 µg $NADP^+$, 1 mg microsomal protein, and 80 nmol of substrate added in 40 µl methanol. The reaction was allowed to proceed for 1 hour in a 37°C shaker water bath in the presence of atmospheric O_2, under yellow light. One volume of acetone was added to terminate the reaction and 2 volumes of ethyl acetate were added to extract substrate and metabolites. The organic layer was separated from the water layer and evaporated under reduced pressure (Büchi Rotavapor-RE/A, Brinkman Instruments, Inc., Westbury, N.Y.). The residue was dissolved in acetone (3 x 2 ml), and the insoluble materials were removed by centrifugation. This acetone solution was then gently evaporated using a Model SVC-100H Speed Vac Concentrator, (Savant Instruments, Inc., Hicksville, N.Y.) and the residue was redissolved in tetrahydrofuran (THF)/methanol (1:1, v/v) for reversed-phase HPLC or in THF/hexane (1:3, v/v) for normal-phase HPLC.

Reaction mixtures for quantitative determinations of the microsomal metabolism of radiolabelled 6-MBaP, BaP, or 6-OHMBaP contained 80 nmol of [^{3}H]6-MBaP, [^{14}C]BaP, or [^{3}H]6-OHMBaP, 0.15 mg 3-MC-micro-

somal protein, 0.3 mg PB-microsomal protein, or 0.4 mg control microsomal protein, and other cofactors as described above in a 1 ml total incubation volume. Incubations were allowed to proceed in (16 x 100 mm) test tubes for 5 or 10 minutes as described above. Three blanks, incubated for either zero time, 5 minutes, or 10 minutes, were prepared by adding boiled microsomes and 1 ml acetone to the reaction mixture prior to incubation. For sample tubes, the enzymatic reactions were stopped by the addition of 1 ml acetone which was evenly dispersed in the tube by mixing for 1 minute. Ethyl acetate (2 ml) was then added to each tube to extract substrate and metabolites, tubes were mixed for 1 minute, and a clear separation of aqueous and organic layers was obtained by centrifugation. The organic layer was pipetted into a separate test tube and dehydrated with anhydrous $MgSO_4$. When the radioactivity of both aqueous and organic phases was determined, labelled material extracted into the organic phase was found to be 98% or greater of the total amount originally added to the incubation mixtures. Samples were then stored in capped tubes at 4°C until processing for up to a week with no perceptible decomposition. Prior to HPLC analysis of metabolites, 1 ml of the organic layer was removed from storage. An unlabelled metabolite mixture prepared from a large scale in vitro incubation of 6-MBaP, BaP, or 6-OHMBaP with rat liver microsomes was added to provide ultraviolet markers for the labelled metabolites. The sample was then gently dried in a vacuum centrifuge, resuspended in up to 25 µl of methanol/THF (1:1 v/v) and injected into the HPLC.

In tabulating the specific activities of metabolite formation, the specific activities of [^{3}H]6-MBaP and [^{3}H]6-OHMBaP metabolites were assumed to be that of the parent compound. The exact amount of tritium

loss due to metabolism of generally labelled [^{3}H]6-MBaP or due to exchange of tritium label with hydrogen atoms in the incubation mixture is not known. Rates of radiolabelled 6-MBaP diol formation are probably quite accurate because label is retained on the molecule during the process of diol formation. However, rates of formation of 6-MBaP phenols and 6-OHMBaP phenols (as metabolites of 6-MBaP) are likely to be somewhat underestimated since a certain but unknown percentage of phenol formation probably takes place via an NIH shift mechanism (20) which results in tritium loss. Estimations of the specific activities of [^{3}H]6-OHMBaP metabolites are thought to be very accurate since no metabolism occurs at the 6-hydroxymethyl group where 6-OHMBaP is specifically labelled. Therefore, any tritium loss would be due only to exchange with hydrogen atoms in the incubation mixture.

Reversed-phase HPLC. Reversed-phase HPLC analyses of labelled or unlabelled 6-MBaP, BaP, or 6-OHMBaP metabolites were performed on a Waters Model 6000A liquid chromatograph equipped with a Model 660 solvent programmer (Waters Associates Inc., Milford Mass.) and fitted with a Vydec C_{18} TP201 5 micron analytical HPLC column (4.6 mm ID x 25 cm; The Separations Group). Metabolites of 6-MBaP, BaP, [^{3}H]6-MBaP, or [^{14}C]BaP were eluted with a 35-minute linear gradient of 50 to 100% methanol in water at a flow rate of 1 ml/minute and were detected by their ultraviolet absorbance at 254 nm (Water Series 440 Absorbance Detector). For increased accuracy in quantitation, the [^{3}H]6-MBaP 4,5-, 7,8-, and 9,10-diol metabolites were also separated with 50% methanol in water at a flow rate of 1.4 ml/minute. These conditions increased the distance between the 9,10- and 7,8-diol peaks as they eluted from the HPLC column. Metabolites of 6-OHMBaP or [^{3}H]6-OHMBaP were eluted with a

35-minute linear gradient of 50 to 90% methanol in water at a flow rate of 1 ml/minute.

For all qualitative work, column eluents were collected directly. For quantitative studies, the eluent was dispensed in approximately 0.3 ml fractions by a Gilson (Middleton, Wisc.) Aliquogel Liquid Column Fractionator. Redi-Solv HP (Beckman) was added to each fraction which was then shaken to ensure dissolution of all material.

Normal-phase HPLC. For nuclear magnetic resonance spectral analysis (NMR), 6-MBaP diol metabolites were separated with a Zorbax SIL HPLC Column (6.2 mm ID x 25 cm; Dupont Instruments Co., Wilmington, Del.) according to methods described in (153).

Radioactivity determination. Radioactivity was determined using a refrigerated Packard Tri-Carb B2450 liquid scintillation counter. Counting efficiency was determined using [^{14}C]toluene or [^{3}H]H_2O standards dissolved in ratios of methanol and water comparable with those used for HPLC. For quantitative analysis of 6-MBaP, BaP, or 6-OHMBaP metabolite formation, a pre-programmed cassette tape inserted into a Hewlett-Packard 9815A calculator was used to convert CPM to picomoles for each ml of incubation mixture after appropriate background subtraction. Radioactivity above baseline which was detected in certain fractions upon analysis of the labelled 6-MBaP, BaP, or 6-OHMBaP incubation blanks was subtracted from the radioactivity contained in the corresponding sample fractions.

Physicochemical properties. Ultraviolet-visible (UV) absorption spectra of metabolites were measured on a Beckman Model 25 spectrophotometer in methanol against a methanol solvent blank. Mass spectral

analysis of metabolites was performed on a Finnigan 4000 gas chromatograph-mass spectrometer-data system (Finnigan Instrument Co., Cincinnati, Ohio) by electron impact with a solid probe at 70 eV and 250°C ionizer temperature. If necessary, 20 to 40 µl of ascorbic acid (0.5% in water) were added prior to mass spectroscopy to prevent decomposition of metabolites. The dihydrodiol metabolites of 6-MBaP in acetone-d_6 with a trace of D_2O were characterized by ^1H NMR on a Bruker WM500 spectrometer. Coupling constants obtained for the 6-MBaP trans diol metabolites were similar to those obtained by the NMR analyses of other PAH trans diols with hydroxyl groups located in sterically hindered regions of their respective parent molecules (137,138,157).

Diacetate derivatization. Due to their labilities, 1-hydroxy- and 3-hydroxy-6-OHMBaP were derivatized to their diacetates prior to mass spectral analysis. Several µg of either phenol were dissolved in 50 µl of anhydrous pyridine. Thirty µl of dimethylaminopyridine and 150 µl of acetic anhydride were then added. The reaction mixture, contained in a tightly capped test tube, was heated at 70°C in a water bath. After 1 hour, 3 ml of ethyl acetate and 1 ml of water were added to the test tube which was then mixed to facilitate extraction of diacetate into the organic layer. The organic layer was washed with water three more times to remove any remaining acid and was then evaporated to dryness and the residue resuspended in 100% methanol. Diacetates were isolated by reversed-phase HPLC using a Dupont Zorbax ODS column (4.6 mm ID x 25 cm) and a 25-minute linear gradient of 60 to 100% methanol in water at a flow rate of 1 ml/minute.

Preparation of metabolites for circular dichroism spectroscopy (CD). Dihydrodiol metabolites of 6-MBaP, BaP, or 6-OHMBaP were isolated by reversed-phase HPLC as described above and were rechromatographed in methanol. The CD spectra were then recorded directly.

Circular dichroism spectroscopy. Optical activity of 6-MBaP, BaP, or 6-OHMBaP dihydrodiol metabolites were determined in methanol in a 1 cm pathlength quartz cell using a Jasco Model 500A spectropolarimeter equipped with a Jasco Model DP-500 data programmer (Jasco Incorp., Easton, MD.). CD spectra were recorded after subtracting appropriate solvent backgrounds and were expressed as ellipticity (ψ_λ in millidegrees at a given wavelength) for a methanol solution that reads 1.0 absorbance in a spectrophotometer at the wavelength of absorption maximum in a cell of 1 cm pathlength. Molecular ellipticity ($[\theta]_\lambda$ in degrees $\cdot$ $cm^2/dmol$) was calculated from ψ_λ using $[\theta]_\lambda = 0.1\varepsilon_{max}\ \psi_\lambda$ and the appropriate diol extinction coefficient.

RESULTS

Effect of the Six-Methyl Group on Metabolite Profiles

In vitro incubations of 6-MBaP and BaP with rat liver microsomes were performed in order to determine the effects of the six methyl group on the types of metabolites formed. In order to achieve the greatest yield of metabolites, 3-MC-microsomes were used in initial studies.

A comparison of the metabolite profiles of 6-MBaP and BaP formed from the incubation of either substrate with 3-MC-microsomes is shown by the chromatograms appearing in Fig. 2 and Fig. 3. Incubations were carried out as described in "Materials and Methods." Metabolites of 6-MBaP or BaP were separated from a concentrated metabolite mixture by reversed-phase HPLC using procedures also described in "Materials and Methods."

The identities of the 6-MBaP metabolites shown in Fig. 2 are (in order of increasing retention time and decreasing polarity): 6-MBaP *trans*-4,5-dihydrodiol (6-MBaP 4,5-diol), 6-MBaP *trans*-9,10-dihydrodiol (6-MBaP 9,10-diol), 6-MBaP *trans*-7,8-dihydrodiol (6-MBaP 7,8-diol), 1-hydroxy-6-hydroxymethyl-BaP (1-OH 6-OHMBaP), 3-hydroxy-6-hydroxymethyl-BaP (3-OH 6-OHMBaP), 6-hydroxymethyl-BaP (6-OHMBaP), 1-hydroxy-6-MBaP (1-OH 6-MBaP), eluted in the left side of the chromatographic peak containing other monohydroxylated metabolites of 6-MBaP (OH 6-MBaP Peak II). The 6-MBaP (structure shown) substrate peak emerged last.

When PB-microsomes were used to metabolize 6-MBaP, two other chromatographic peaks were obtained which were not detected in the profile of metabolites formed by 3-MC-microsomes. The radiogram depicted in Fig. 5 confirmed the elution positions of these two peaks whose retention times are indicated in Fig. 2 by dashed lines. The first

peak was positively identified as 12-hydroxy 6-MBaP (12-OH 6-MBaP), while the second was tentatively identified as a monohydroxylated metabolite of 6-MBaP (OH 6-MBaP Peak I). 12-Hydroxy 6-MBaP was also detected when control microsomes were used.

The radiograms shown in Fig. 4 and Fig. 5 were obtained after HPLC separation and quantitative analysis of the metabolites of [^{3}H]6-MBaP formed by 3-MC- or PB-microsomes using procedures described in "Materials and Methods." Chromatographic conditions were identical to those used to separate the metabolites formed from unlabelled substrate (see Fig. 2). The radiogram obtained upon analysis of [^{3}H]6-MBaP metabolism by control microsomes was similar to that shown for PB-microsomes and is not presented. Metabolite peaks indicated in the radiogram in Fig. 4 or Fig. 5 correspond to particular chromatographic peaks shown or indicated in Fig. 2.

Identical conditions were used to separate the metabolites of BaP shown in the chromatogram in Fig. 3. They are (in order of increasing retention time and decreasing polarity): BaP 9,10-dihydrodiol (BaP 9,10-diol), BaP 4,5-dihydrodiol (BaP 4,5-diol), BaP 7,8-dihydrodiol (BaP 7,8-diol), BaP 1,6-quinone, BaP 3,6-quinone, BaP 6,12-quinone, 9-hydroxy BaP (9-OH BaP), 7-hydroxy BaP (7-OH BaP), 1-hydroxy BaP (1-OH BaP), and 3-hydroxy BaP (3-OH BaP). The BaP (structure shown) substrate peak was eluted last. A 5-hydroxy BaP (5-OH BaP) synthetic standard was found to coelute with the BaP 6,12-quinone.

There are obvious similarities and differences in the metabolite profiles obtained from the metabolism of 6-MBaP and BaP (Figs. 2 and 3). Both substrates are metabolized to three diol metabolites (4,5-, 7,8-, and 9,10-diols) which differ in their elution order and retention times.

Fig. 2. Reversed-phase HPLC profile of 6-MBaP metabolites formed from 3-MC-microsomes. Metabolites were eluted from a Vydec C_{18} HPLC column with a 35-minute linear gradient of 50 to 100% methanol in water at a flow rate of 1 ml/minute. The dashed lines indicate the retention times of two 6-MBaP metabolites formed when other microsomal enzyme preparations were used. See text for discussion. Ultraviolet-visible absorption spectral analysis of the left side of OH 6-MBaP Peak II provided evidence that it contained the 6-MBaP 1-phenol metabolite of 6-MBaP (1-OH 6-MBaP, indicated by the arrow).

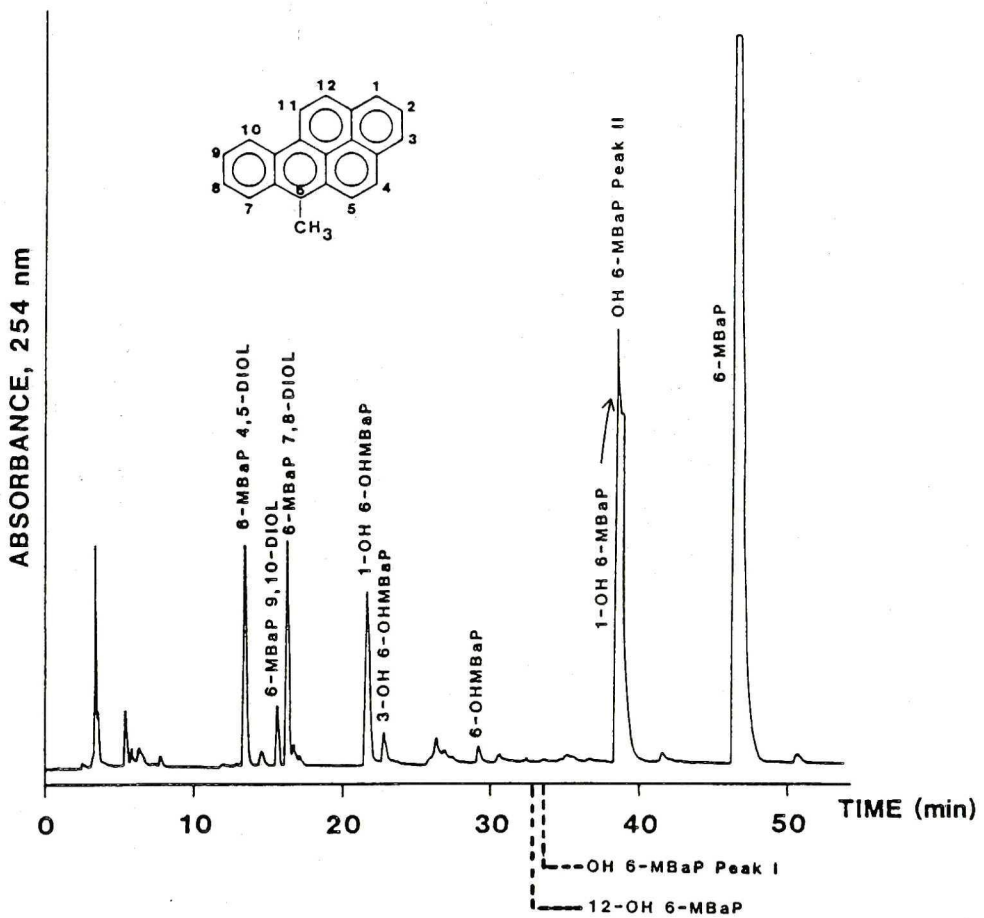

ABSORBANCE, 254 nm
6-MBaP 4,5-DIOL
6-MBaP 9,10-DIOL
6-MBaP 7,8-DIOL
1-OH 6-OHMBaP
3-OH 6-OHMBaP
6-OHMBaP
1-OH 6-MBaP
OH 6-MBaP Peak II
6-MBaP
12
1
2
3
4
5
6
7
8
9
10
11
CH3
0
10
20
30
40
50
TIME (min)
OH 6-MBaP Peak I
12-OH 6-MBaP

Fig. 3. Reversed-phase HPLC profile of BaP metabolites formed from 3-MC-microsomes. Separation conditions were identical to those used for 6-MBaP metabolite separation as described in Fig. 2.

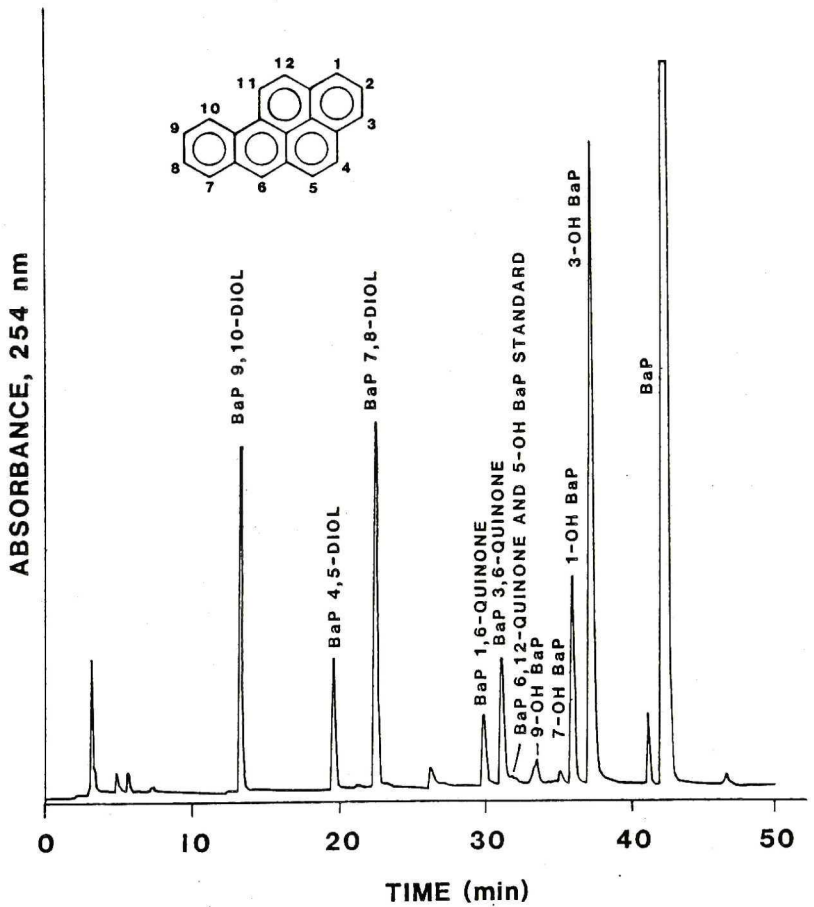

ABSORBANCE, 254 nm
BaP 9,10-DIOL
BaP 4,5-DIOL
BaP 7,8-DIOL
BaP 1,6-QUINONE
BaP 3,6-QUINONE
BaP 6,12-QUINONE AND 5-OH BaP STANDARD
9-OH BaP
7-OH BaP
1-OH BaP
3-OH BaP
BaP
0
10
20
30
40
50
TIME (min)

Fig. 4. Axis A. The profile of metabolites formed from the metabolism of [^{3}H]6-MBaP by 3-MC-microsomes after correction for background radioactivity. Axis B. Corresponding zero-time incubation blank after correction for background radioactivity. Similar results were obtained upon analysis of the 10-minute incubation blank. Relevant procedures are described in "Materials and Methods." Reversed-phase chromatographic conditions were identical to those described in Fig. 2.

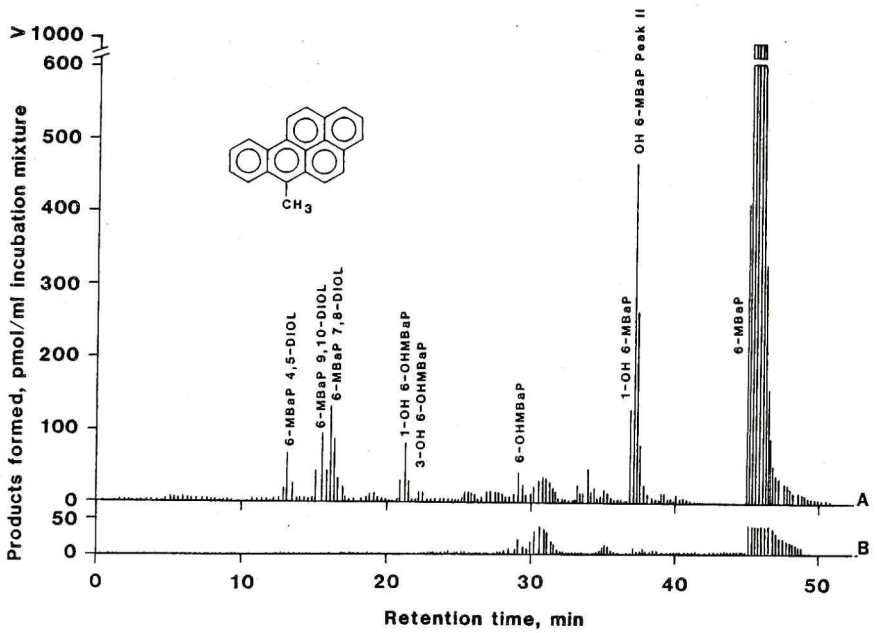
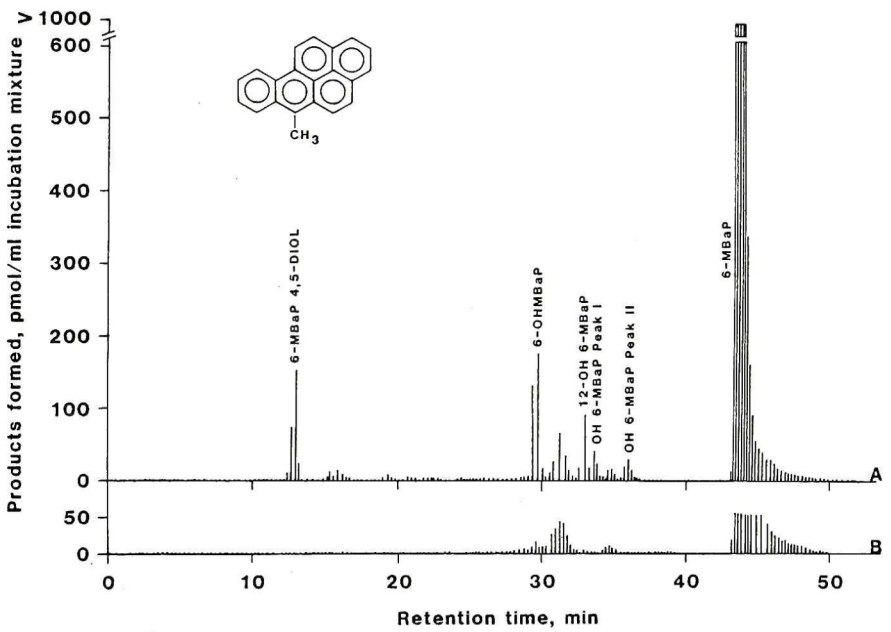

Products formed, pmol/ml incubation mixture
>1000
600
500
400
300
200
100
0
50
0
A
B
0
10
20
30
40
50
Retention time, min
CH3
6-MBaP 4,5-DIOL
6-MBaP 9,10-DIOL
6-MBaP 7,8-DIOL
1-OH 6-OHMBaP
3-OH 6-OHMBaP
6-OHMBaP
1-OH 6-MBaP
OH 6-MBaP Peak II
6-MBaP

Fig. 5. Axis A. The profile of metabolites formed from the metabolism of [^{3}H]6-MBaP by PB-microsomes after correction for background radioactivity. Axis B. Corresponding zero-time incubation blank after correction for background radioactivity. Similar results were obtained upon analysis of the 10-minute incubation blank. Relevant procedures are described in "Materials and Methods." Reversed-phase chromatographic conditions were identical to those described in Fig. 2.


Products formed, pmol/ml incubation mixture
1000
600
500
400
300
200
100
0
50
0
6-MBaP 4,5-DIOL
6-OHMBaP
12-OH 6-MBaP Peak I
OH 6-MBaP Peak II
6-MBaP
CH3
A
B
0
10
20
30
40
50
Retention time, min

In a previous study conducted in this laboratory (153), a two-step process using both reversed- and normal-phase HPLC was necessary to achieve separation of the 6-MBaP 7,8- and 9,10-diols. However, in this study, a one-step baseline separation of the two diols was achieved (as shown in Fig. 2) when a small-volume amount of material was applied to the Vydec C_{18} HPLC column. This column contains smaller particle size packing material (5 micron) than did the column used in the earlier investigation (μBondapak (Waters) C_{18} column with 10 micron particle size). As a result of the decrease in particle size, the Vydec column had about five times the number of theoretical plates calculated for the μBondapak column.

Monohydroxylation at various positions of 6-MBaP and BaP resulted in a number of phenolic metabolites. Although resolution of OH 6-MBaP Peak II is poor, and attempts to resolve the peak more effectively were never successful, UV absorption spectral analysis indicated that one component of the peak is 1-OH 6-MBaP (Fig. 6), while 3-OH and 9-OH 6-MBaP are thought to be the other predominant components. An effective separation of BaP 1-, 3-, 5-, and 7-phenols was obtained using the Vydec C_{18} column (Fig. 3). This is a considerable improvement over the one-step separation of these compounds reported previously (17,26,33) wherein the 1-, 3-, 5-, and 7-phenols coeluted in a single peak.

Due to hydroxylation of the methyl group, a set of metabolites unique to the metabolism of 6-MBaP was formed (compare Figs. 2 and 3). These metabolites include 6-OHMBaP and the 1- and 3-phenols of 6-OHMBaP.

Quinones were not detected as metabolites of 6-MBaP. Unlike BaP, the methyl group of 6-MBaP appears to block the formation of 1,6-, 3,6,- and 6,12-quinones which appear in the BaP metabolite profile.

Identification of 6-MBaP Metabolites

6-MBaP metabolites were characterized by several techniques including UV absorption spectral analysis, mass spectral analysis, and nuclear magnetic resonance spectral analysis (NMR).

UV absorption spectral analysis. Tentative identification of certain metabolites (the three 6-MBaP diols, the 6-OHMBaP 1- and 3-phenols, and the 6-MBaP 1- and 12-phenols) by UV absorption spectral analysis was made possible by their structural similarities to the corresponding BaP metabolite or synthetic standards. The absorption spectra of 6-MBaP metabolites dissolved in methanol were obtained as described in "Materials and Methods" and were compared with the analagous BaP standards as shown in Figs. 6-12. Absolute structures for the predominant enantiomers of the 6-MBaP and BaP diol metabolites and the structures for other 6-MBaP and BaP metabolites are also given in Figs. 6-12.

It can be seen that the UV absorption spectra for 6-MBaP metabolites and corresponding BaP standards are virtually identical except for a slight bathochromic shift of 1-5 nm in the 6-MBaP metabolite spectra. Tentative identification of the 6-OHMBaP metabolite of 6-MBaP was made by comparing the UV absorption spectrum of the metabolite to that of a synthetic 6-OHMBaP standard. Confirmation of the structure of 6-OHMBaP was obtained by mass spectral analysis of the metabolite.

In the presence of alkali, aromatic phenols show a characteristic bathochromic shift in their UV absorption spectra due to resonance stabilization of the phenoxide anion (154). Methanolic solutions of the metabolites tentatively identified as the 6-OHMBaP 1- and 3-phenols, and the 6-MBaP 1-phenol, showed this red shift upon the addition of

Fig. 6. The ultraviolet-visible absorption spectra of 1-OH 6-OHMBaP (a metabolite of 6-MBaP and 6-OHMBaP) in methanol (——————) or in the presence of alkali (— — — —) are compared with those of 1-OH BaP (a synthetic standard) in methanol (—·—·—) or in the presence of alkali (—···—···). The spectra for a metabolite of 6-MBaP tentatively identified as 1-OH 6-MBaP in methanol (·····) and in the presence of alkali are also shown (•—•—•—•). See text for discussion.

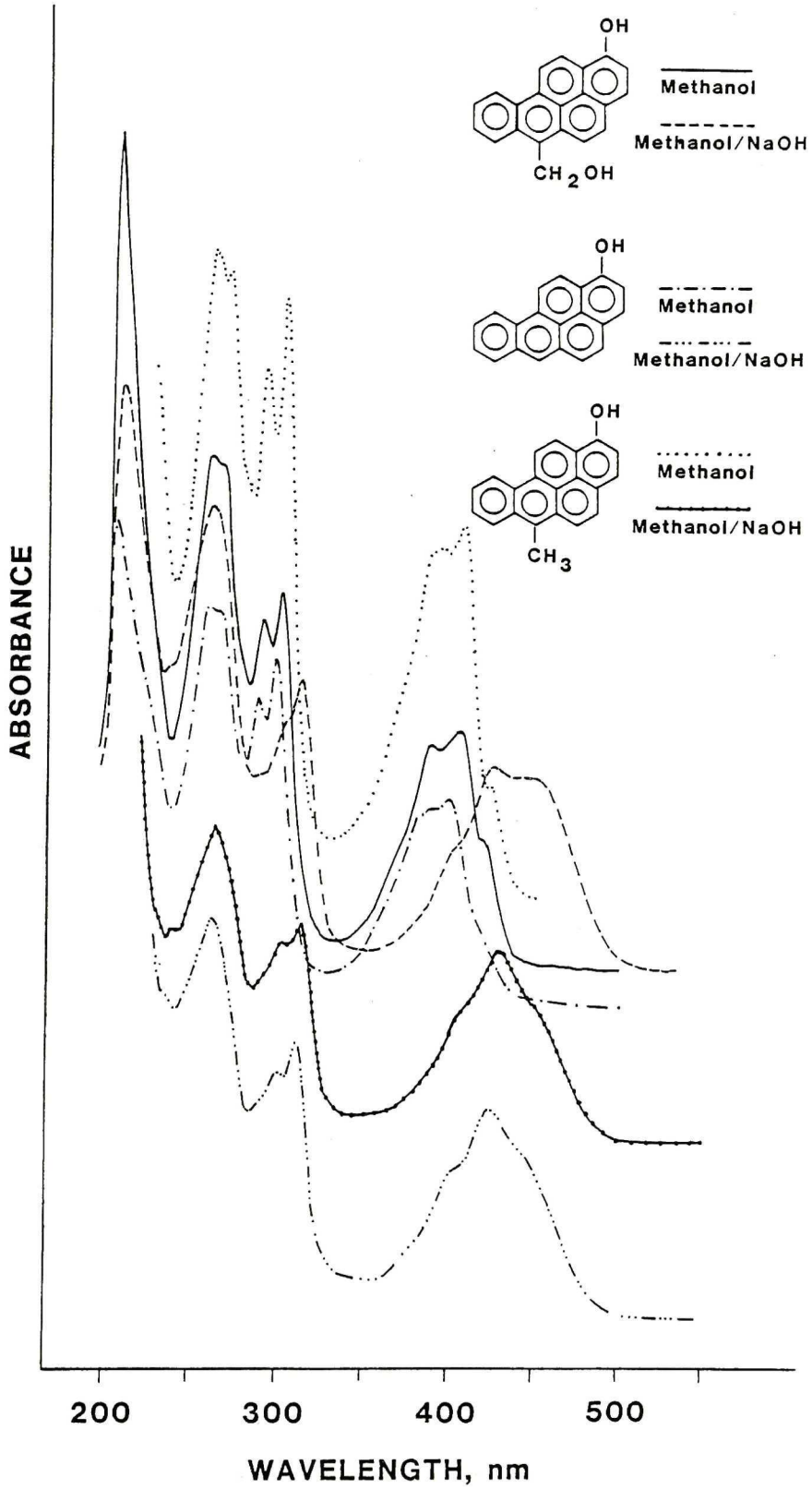

OH
CH2OH
Methanol
Methanol/NaOH
OH
Methanol
Methanol/NaOH
OH
CH3
Methanol
Methanol/NaOH
ABSORBANCE
200
300
400
500
WAVELENGTH, nm

Fig. 7. Ultraviolet-visible absorption spectra of 6-MBaP trans-4,5-dihydrodiol (a metabolite of 6-MBaP; solid line) and BaP trans-4,5-dihydrodiol (a metabolite standard; dashed line). The major enantiomers of the 6-MBaP and the BaP trans-4,5-dihydrodiol metabolites formed stereoselectively from the in vitro metabolism of 6-MBaP or BaP by rat liver microsomes have [4R,5R] absolute stereochemistry, as indicated by the structures shown. Substituents attached by triangle bonds (▼) are oriented towards the viewer, while substituents attached by dashed bonds (⩧) are oriented away from the viewer.

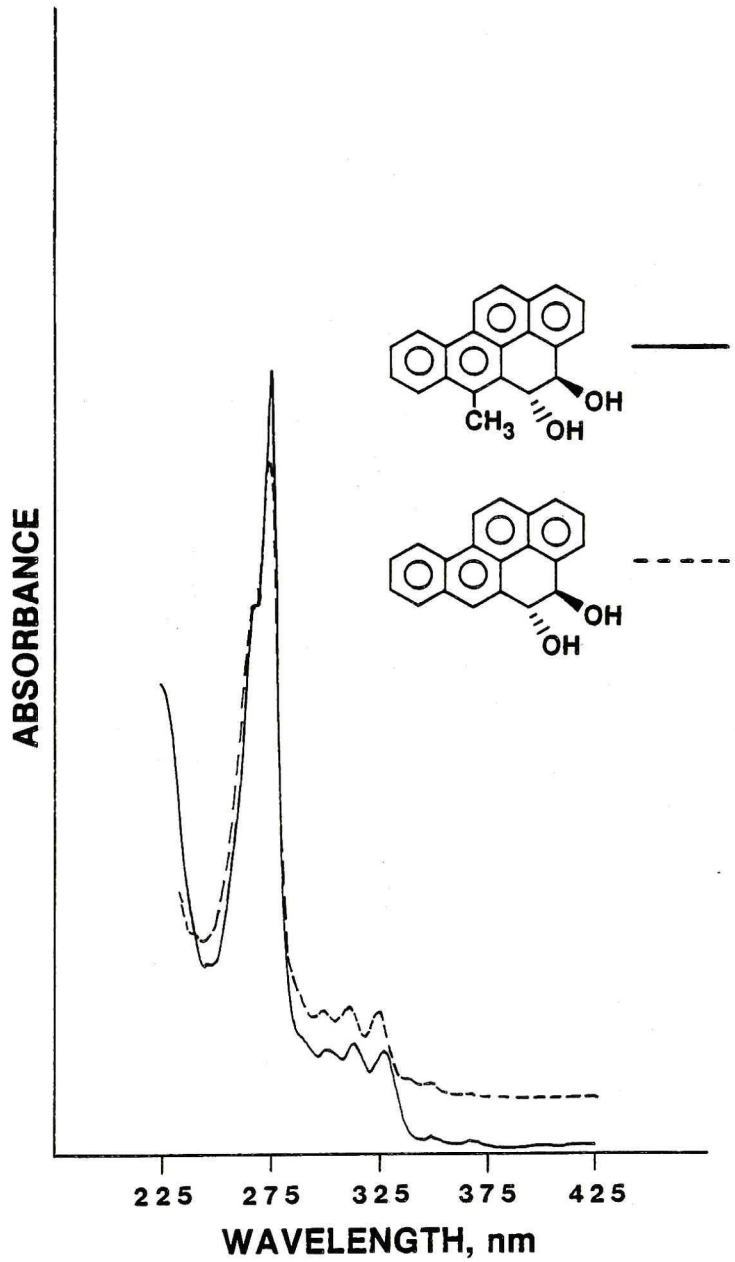

ABSORBANCE
OH
CH3
OH
OH
OH
225
275
325
375
425
WAVELENGTH, nm

Fig. 8. Ultraviolet-visible absorption spectra of 6-MBaP trans-7,8-dihydrodiol (a metabolite of 6-MBaP; solid line) and BaP trans-7,8-dihydrodiol (a metabolite standard; dashed line). The major enantiomers of the 6-MBaP and the BaP trans-7,8-dihydrodiol metabolites formed stereoselectively from the in vitro metabolism of 6-MBaP or BaP by rat liver microsomes have [7R,8R] absolute stereochemistry, as indicated by the structures shown. Substituents attached by triangle bonds (▼) are oriented towards the viewer, while substituents attached by dashed bonds (⥤) are oriented away from the viewer.

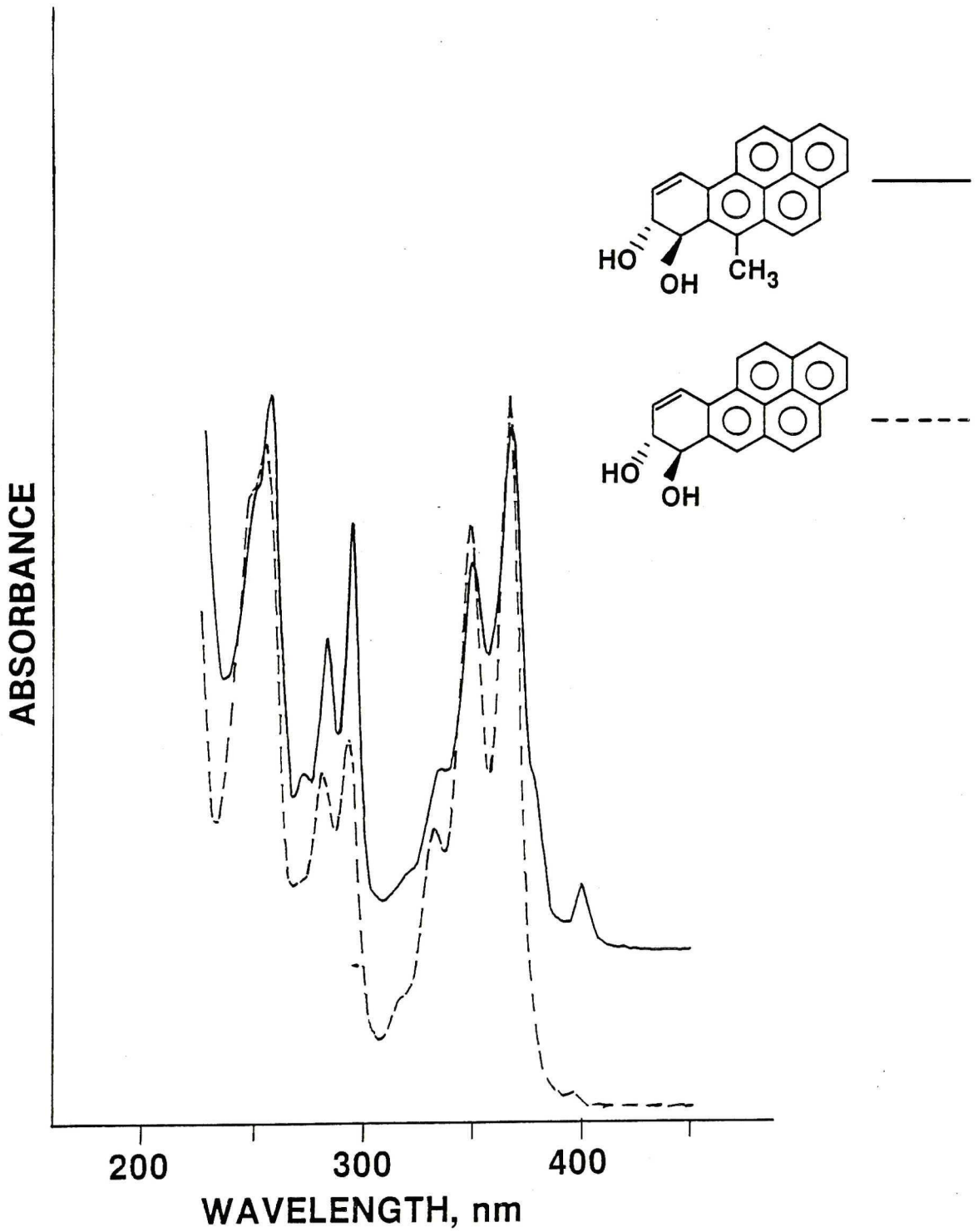

ABSORBANCE
200
300
400
WAVELENGTH, nm
HO
OH
CH3
HO
OH

Fig. 9. Ultraviolet-visible absorption spectra of 6-MBaP trans-9,10-dihydrodiol (a metabolite of 6-MBaP; solid line) and BaP trans-9,10-dihydrodiol (a metabolite standard; dashed line). The major enantiomers of the 6-MBaP and the BaP trans-9,10-dihydrodiol metabolites formed stereoselectively from the in vitro metabolism of 6-MBaP or BaP by rat liver microsomes have [9R,10R] absolute stereochemistry, as indicated by the structures shown. Substituents attached by triangle bonds (▌) are oriented towards the viewer, while substituents attached by dashed bonds (≡) are oriented away from the viewer.

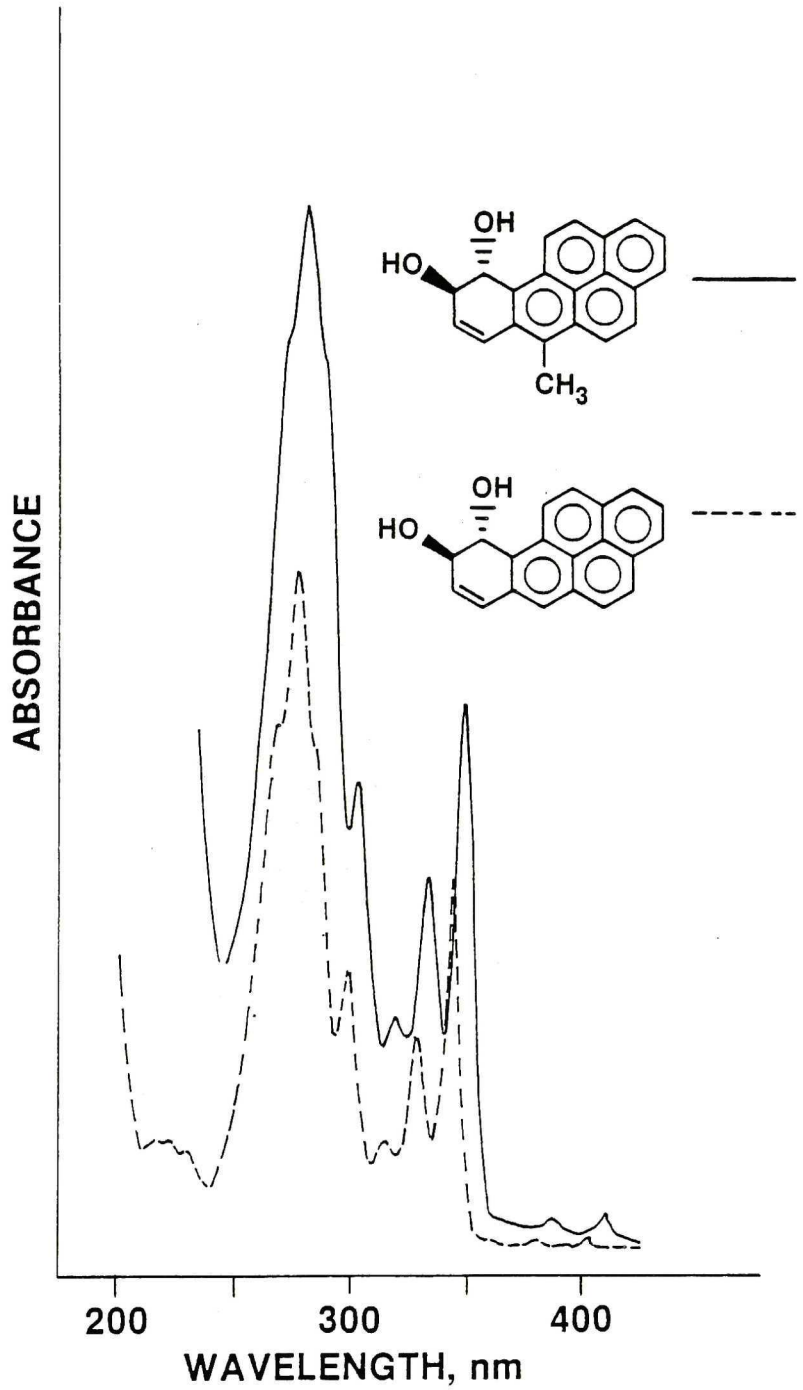

OH
HO
CH3
OH
HO
ABSORBANCE
200
300
400
WAVELENGTH, nm

Fig. 10. The ultraviolet-visible absorption spectra of 3-OH 6-OHMBaP (a metabolite of 6-MBaP and 6-OHMBaP) in methanol (————) and in the presence of alkali (– – – –) are compared with those of 3-OH BaP (a synthetic standard) in methanol (—·—·—) and in the presence of alkali (·····).

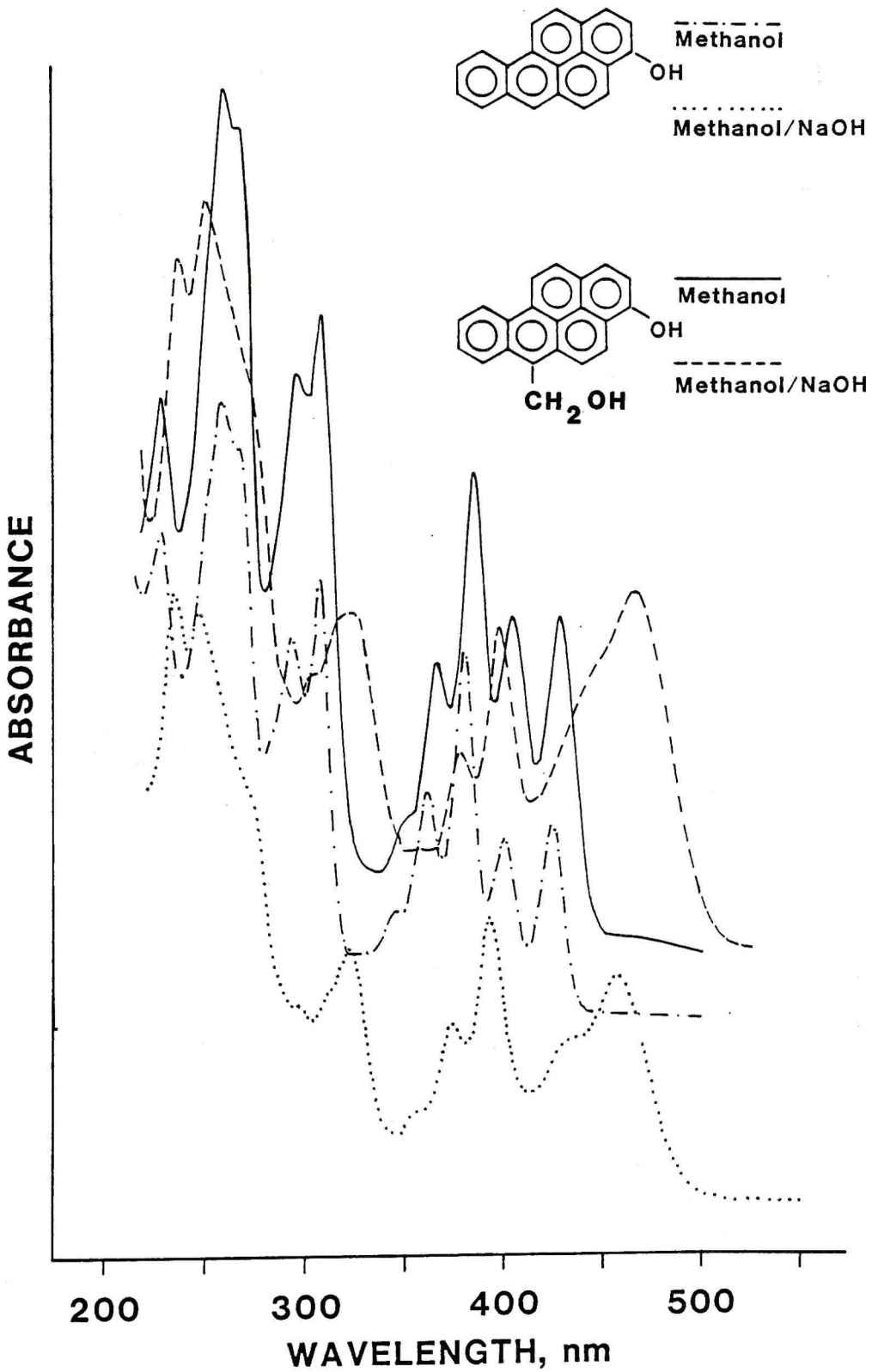

Methanol
OH
Methanol/NaOH
Methanol
OH
CH2OH
Methanol/NaOH
ABSORBANCE
200
300
400
500
WAVELENGTH, nm

Fig. 11. Ultraviolet-visible absorption spectra of 4-OH and 5-OH BaP (synthetic standards) in methanol (4-OH BaP, ·····—····· ; 5-OH BaP, – – – –) and in the presence of alkali (4-OH BaP, •••••• ; 5-OH BaP, —·—·—) are compared with those of a compound tentatively identified as a monohydroxy metabolite of 6-MBaP formed by PB-microsomes (OH 6-MBaP Peak I in methanol, ——— , and in the presence of alkali, ·····).

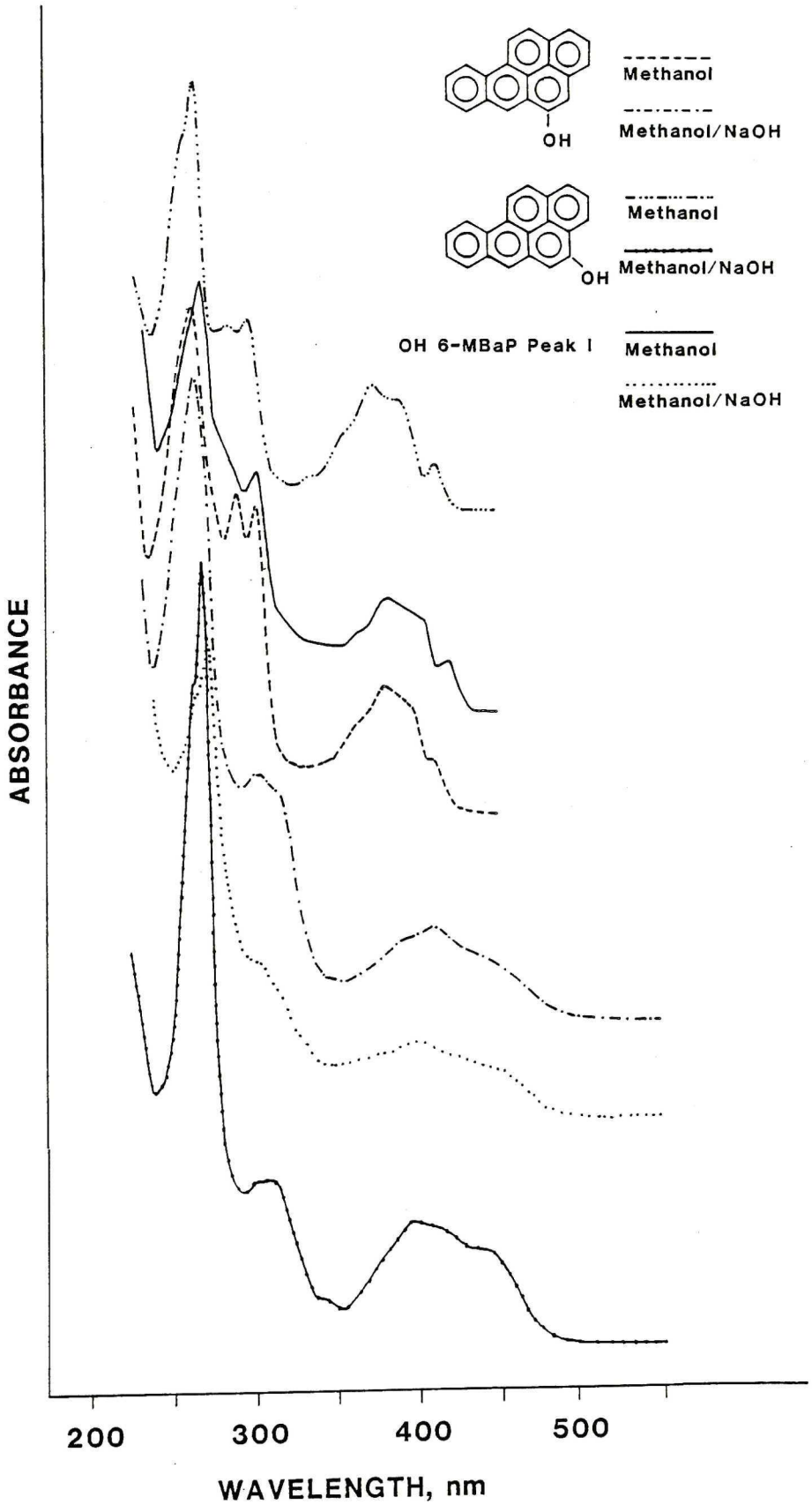
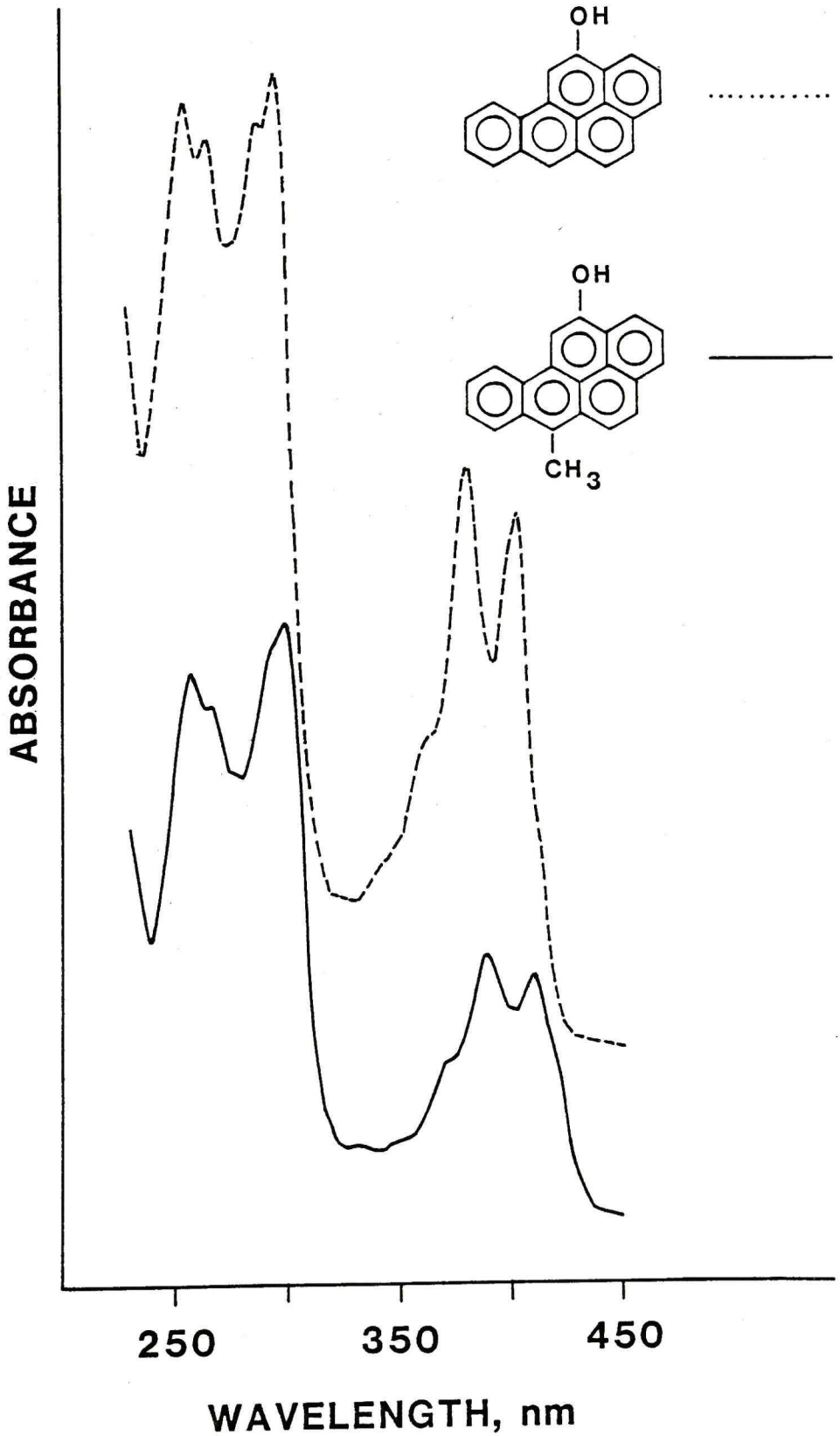

OH
Methanol
Methanol/NaOH
OH
Methanol
Methanol/NaOH
OH 6-MBaP Peak I
Methanol
Methanol/NaOH
ABSORBANCE
200
300
400
500
WAVELENGTH, nm

Fig. 12. Ultraviolet-visible absorption spectra of 12-OH 6-MBaP (a metabolite of 6-MBaP formed by PB- and control microsomes; solid line) and 12-OH BaP (a synthetic standard; dashed line).


OH
OH
CH3
ABSORBANCE
250
350
450
WAVELENGTH, nm

0.1 N NaOH, as did their corresponding BaP metabolite standards (Figs. 6 and 10). In the presence of alkali, the UV absorption spectrum of the metabolite of 6-MBaP labelled OH 6-MBaP Peak I in Fig. 2 also displayed a bathochromic shift, which indicated that it was a phenol (Fig. 11). The compound's chromatographic properties on the Vydec C_{18} column (Fig. 2) more closely resembled those of the 6-MBaP phenol metabolites than those of the relatively more polar 6-OHMBaP phenol metabolites. However, because the UV absorption spectrum of OH 6-MBaP Peak I in methanol resembled those of both 4-OH BaP and 5-OH BaP (Fig. 11), a more complete tentative identification was not possible. The compound is a relatively minor metabolite of 6-MBaP detected only when PB-microsomes were used.

Mass spectral analysis. The identities tentatively conferred on the metabolites of 6-MBaP by UV absorption spectral analysis were confirmed by mass spectral analysis of the metabolites as described in "Materials and Methods."

Molecular weights of the three 6-MBaP diol metabolites (Figs. 13-15) were confirmed by mass ions at m/z 300 and characteristic fragment ions at m/z 282 (loss of H_2O), 267 (loss of H_2O and CH_3), 264 (loss of $2H_2O$), 252 (loss of two OH groups and CH_2), 250 (loss of H_2O, OH, and CH_3), and at 239 and 226 (breakup of ring structure).

6-OHMBaP, with mass ion at m/z 282, gave fragment ions at m/z 265, 253, 252, and 251 indicating fragmentation of the hydroxymethyl group (Fig. 16).

Due to the lability of the 6-OHMBaP 1- and 3-phenols, diacetate derivatives were made of these two metabolites in an effort to increase their stability. Details of the preparation of these diacetates and

Fig. 13. Mass spectrum of the trans-4,5-dihydrodiol metabolite of 6-MBaP. The mass ion is indicated as (M+) and characteristic ion fragments are labelled. The [4R,5R] absolute stereochemistry of the major enantiomer of the diol is indicated by the structure shown.

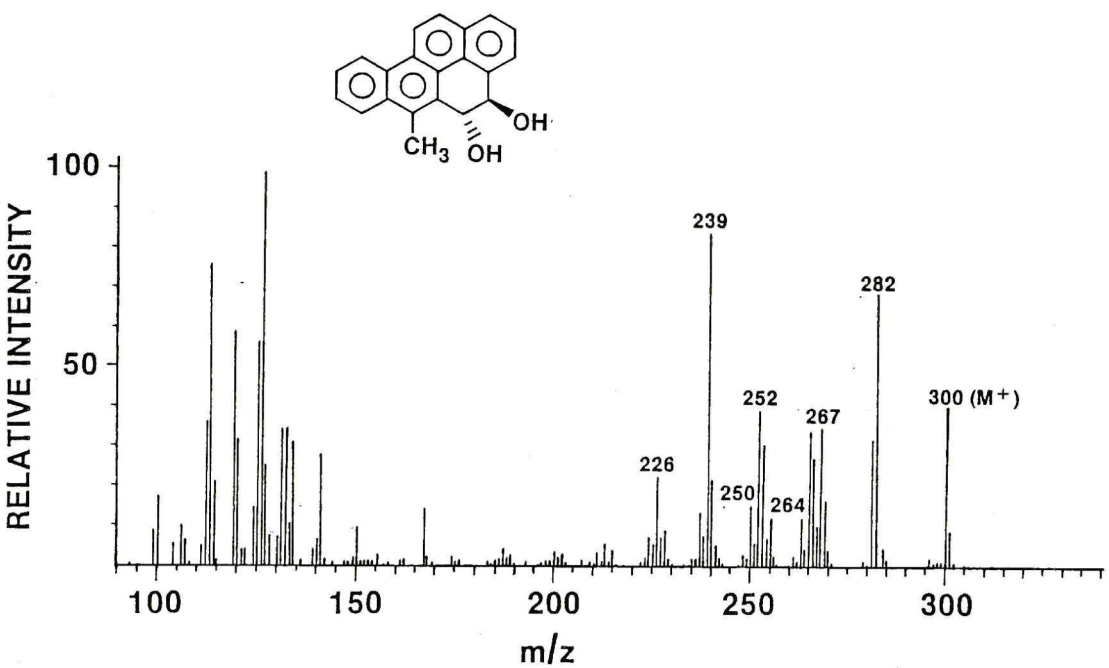

OH
CH3 OH
100
50
RELATIVE INTENSITY
239
282
252
267
300 (M+)
226
250
264
100
150
200
250
300
m/z

Fig. 14. Mass spectrum of the trans-7,8-dihydrodiol metabolite of 6-MBaP. The mass ion is indicated as (M+) and characteristic ion fragments are labelled. The [7R,8R] absolute stereochemistry of the major enantiomer of the diol is indicated by the structure shown.

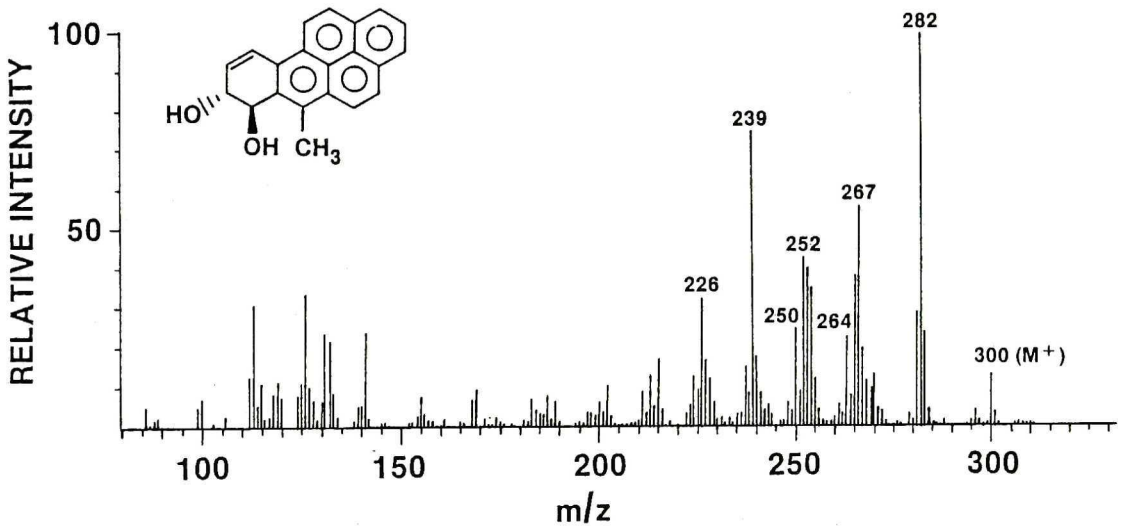

RELATIVE INTENSITY
100
50
HO
OH
CH3
282
239
267
252
226
250
264
300 (M+)
100
150
200
250
300
m/z

Fig. 15. Mass spectrum of the trans-9,10-dihydrodiol metabolite of 6-MBaP. The mass ion is indicated as (M+) and characteristic ion fragments are labelled. The [9R,10R] absolute stereochemistry of the major enantiomer of the diol is indicated by the structure shown.

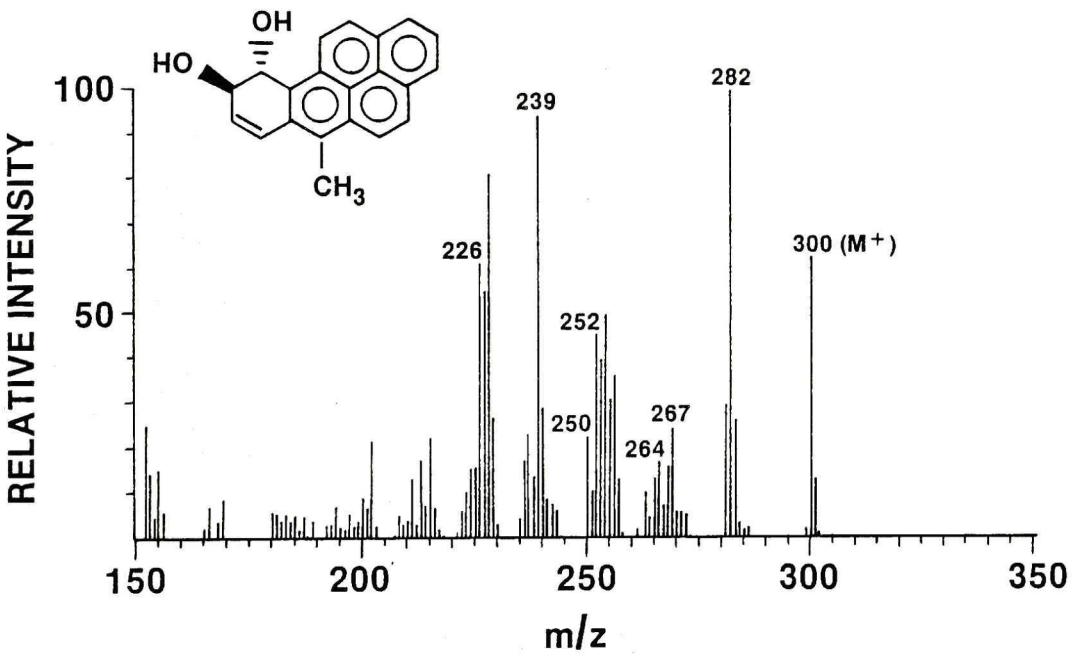

OH
HO
CH3
100
50
RELATIVE INTENSITY
226
239
252
250
264
267
282
300 (M+)
150
200
250
300
350
m/z

Fig. 16. Mass spectrum of the 6-OHMBaP metabolite of 6-MBaP. The mass ion is indicated as (M+) and characteristic ion fragments are labelled.

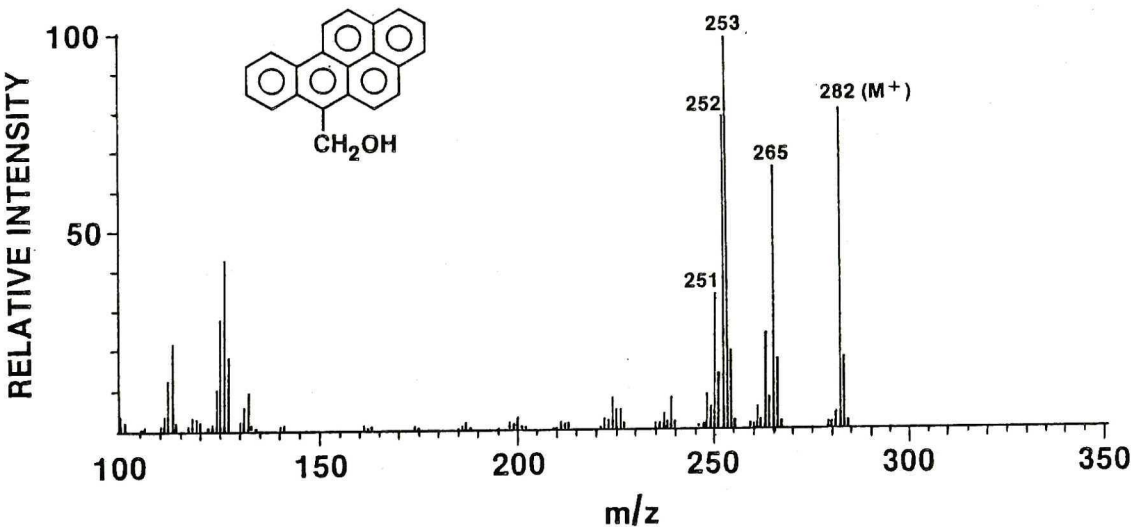

RELATIVE INTENSITY
100
50
CH2OH
253
252
251
265
282 (M+)
100
150
200
250
300
350
m/z

Fig. 17. Mass spectrum of 1-OH 6-OHMBaP (a metabolite of both 6-MBaP and 6-OHMBaP) as a diacetate. The mass ion is indicated as (M+) and characteristic ion fragments are labelled. The structure of 1-OH 6-OHMBaP before and after derivatization is shown. DMAP is the abbreviation for dimethylaminopyridine.

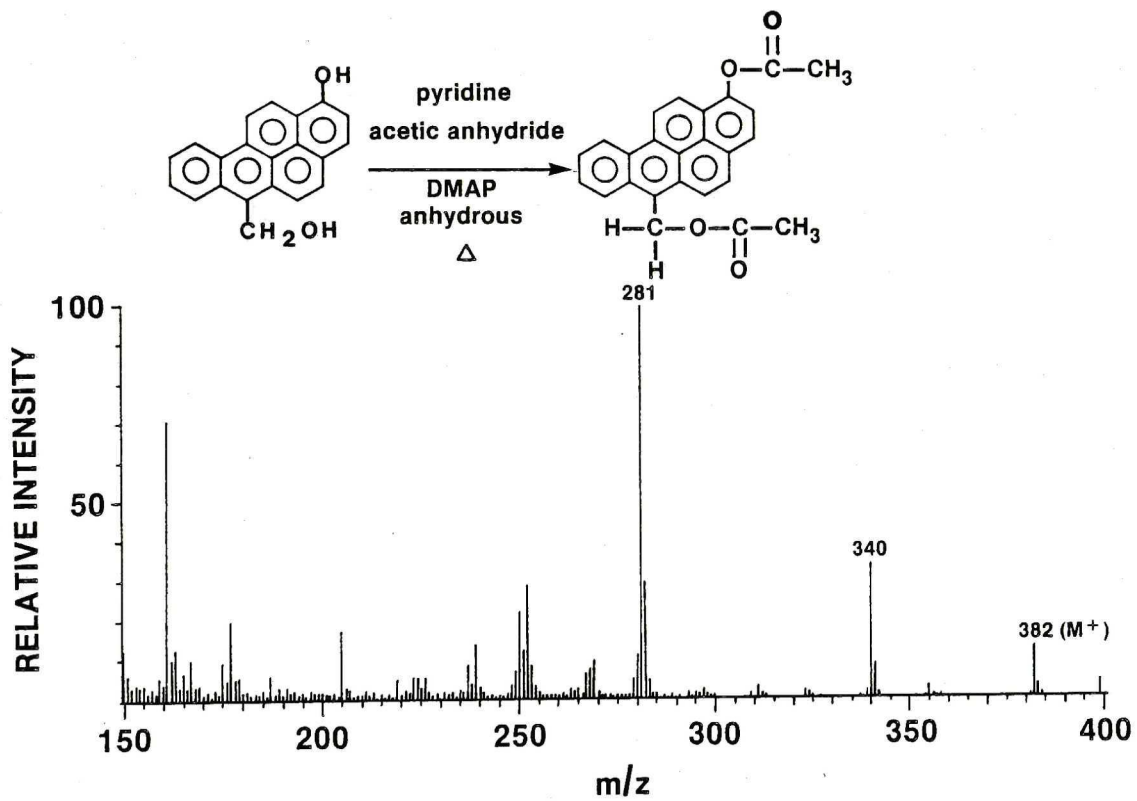
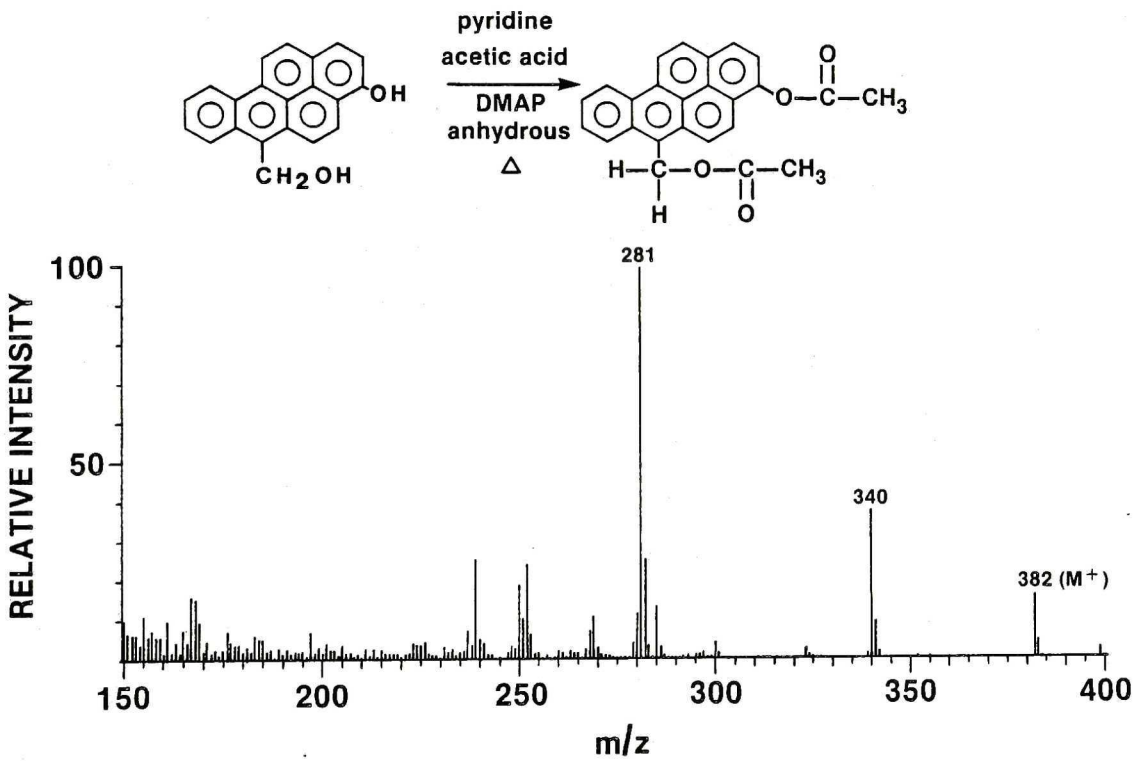

OH
CH2OH
pyridine
acetic anhydride
DMAP
anhydrous
△
O
O—C—CH3
H—C—O—C—CH3
H
O
RELATIVE INTENSITY
100
50
281
340
382 (M+)
150
200
250
300
350
400
m/z

Fig. 18. Mass spectrum of 3-OH 6-OHMBaP (a metabolite of both 6-MBaP and 6-OHMBaP) as a diacetate. The mass ion is indicated as (M+) and characteristic ion fragments are labelled. The structure of 3-OH 6-OHMBaP before and after derivatization is shown. DMAP is the abbreviation for dimethylaminopyridine.


pyridine
acetic acid
DMAP
anhydrous
△
OH
CH2OH
O—C—CH3
H—C—O—C—CH3
H
O
RELATIVE INTENSITY
100
50
281
340
382 (M+)
150
200
250
300
350
400
m/z

their structures appear in "Materials and Methods" and Figs. 17 and 18 respectively. Conversion of either the 1- or 3-hydroxy group of the 6-OHMBaP 1- and 3-phenols to an acetate destroys the phenolic character of the molecule; therefore, the UV absorption spectra of both the diacetates of the 6-OHMBaP 1- and 3-phenols were similar to that of 6-OHMBaP as anticipated. Mass spectral analysis (Figs. 17 and 18) confirmed the structures of the two diacetates with mass ions at m/z 382 and ion fragments at 340 and 281, indicative of the loss of CH_2CO and the loss of CH_2CO and CH_3COO respectively.

A predominant mass ion at m/z 282 confirmed the molecular weight of the 6-MBaP 12-phenol metabolite (Fig. 20). Characteristic ion fragments appeared at m/z 265 (loss of OH), 267 (loss of CH_3), 250 (loss of OH and CH_3), and 252 (loss of CH_2O). Analysis of the mixture of 6-MBaP phenols (OH 6-MBaP Peak II) showed the same mass ion and a similar fragmentation pattern (Fig. 19). This evidence supported the UV absorption spectral data which indicated that one of the components of the 6-MBaP phenol metabolite mixture obtained with 3-MC-microsomes was the 6-MBaP 1-phenol. Mass spectral analysis of OH 6-MBaP Peak I was unsuccessful due to the lability of the metabolite. Attempts to form an acetate derivative of the compound were also unsuccessful.

Geometry and Conformation of the 6-MBaP Diol Hydroxyl Groups

NMR spectral analysis was used to definitively establish the geometry and conformation of the hydroxyl groups of the three 6-MBaP diols. The proton assignments are as follows:

6-MBaP trans-4,5-diol (acetone-d_6 with trace of D_2O): 5.20 (d, H_4) and 5.72 ppm (d, H_5); $J_{4,5}$ = 3.0 Hz.

6-MBaP trans-7,8-diol (acetone-d_6 with trace of D_2O): 5.40

Fig. 19. Mass spectrum of monohydroxy metabolites of 6-MBaP (OH 6-MBaP Peak II). The mass ion is indicated as (M+) and characteristic ion fragments are labelled. See text for discussion.

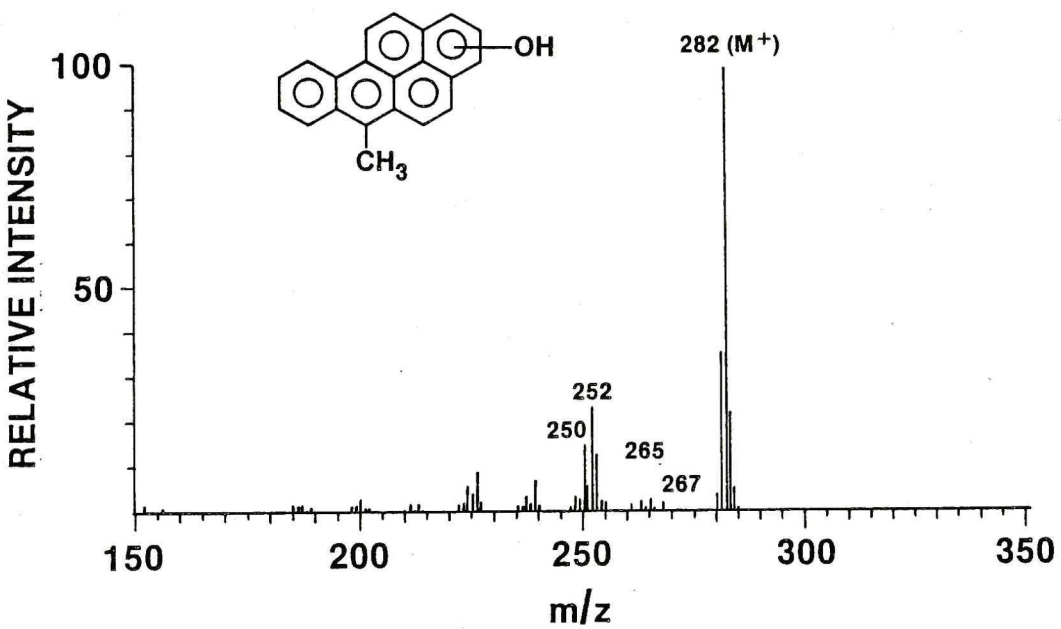
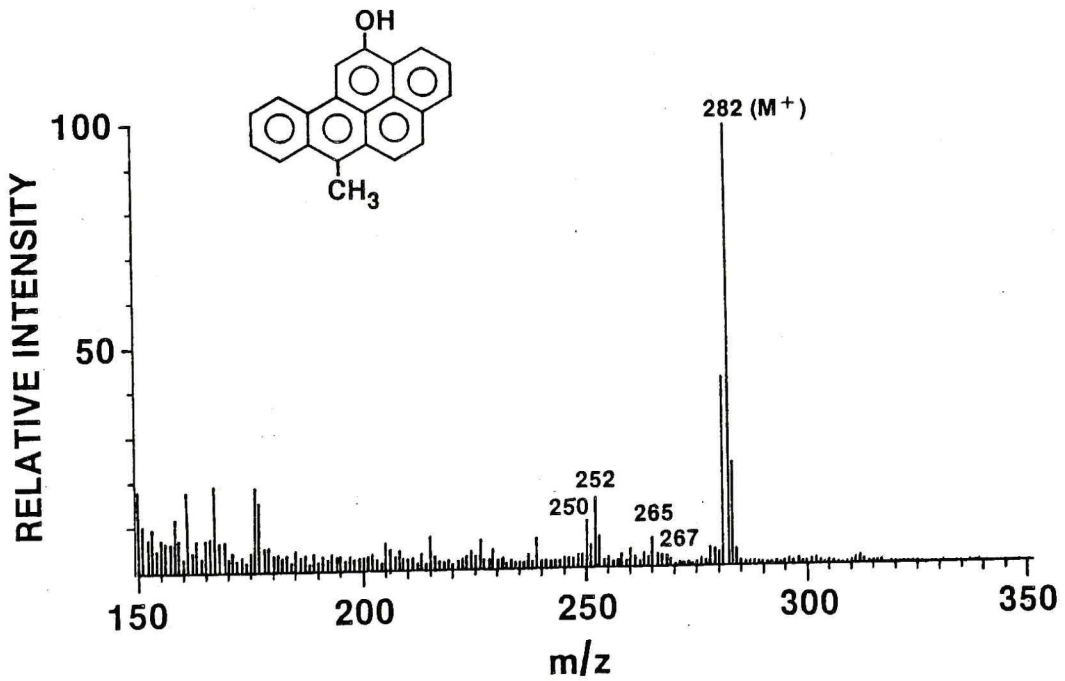

OH
CH3
282 (M+)
252
250
265
267
100
50
RELATIVE INTENSITY
150
200
250
300
350
m/z

Fig. 20. Mass spectrum of the 12-OH metabolite of 6-MBaP. The mass ion is indicated as (M+) and characteristic ion fragments are labelled.


OH
CH3
282 (M+)
252
250
265
267
100
50
RELATIVE INTENSITY
150
200
250
300
350
m/z

(app. s, H_7), 4.49 (app. d, H_8), 6.49 (dd, H_9), and 7.79 ppm (d, H_{10}); $J_{7,8}$ = 1.7, $J_{8,9}$ = 5.5, and $J_{9,10}$ = 9.9 Hz.

6-MBaP trans-9,10-dihydrodiol (acetone-d_6 with trace of D_2O): 4.49 (app. d, H_9), 5.70 (app. s, H_{10}), 7.38 (d, H_7), and 6.54 ppm (dd, H_8); $J_{9,10}$ = 2.0 Hz, $J_{7,8}$ = 9.9, and $J_{8,9}$ = 5.5 Hz.

The small coupling constants $J_{4,5}$ = 3.0 Hz, $J_{7,8}$ = 1.7 Hz, and $J_{9,10}$ = 2.0 Hz for the 6-MBaP trans-4,5-, 7,8-, and 9,10-diols respectively indicate that the hydroxyl groups of these three diols are in the trans configuration and quasi-diaxial conformation (137,138,157). Both the 6-MBaP 7,8- and 9,10-diols have a $J_{8,9}$ coupling constant of 5.5 Hz which further supports their quasi-diaxial conformational assignments.

Comparison of the conformations of the 6-MBaP and BaP trans diols yields similarities and striking differences. The hydroxyls of both the 6-MBaP and BaP trans 9,10-diols preferentially adopt the quasi-diaxial (AA) conformation due to their locations in the sterically hindered bay regions of their respective parent hydrocarbons.

However, while the BaP trans-4,5- and 7,8-diols preferentially adopt the quasi-diequatorial (EE) conformation, it appears that the presence of the methyl group at position six sterically forces the hydroxyl groups of the 6-MBaP 4,5- and 7,8-diols to preferentially adopt the AA conformation (see Table 1).

Quantitative Comparison of the Metabolism of 6-MBaP and BaP by 3-MC-, PB-, and Control Liver Microsomes

A series of experiments was carried out in order to quantitatively compare the effects of the six methyl group on the metabolism of 6-MBaP and BaP and to determine any differences in the regioselectiv-

TABLE 1

Conformational Preferences of 6-MBaP and BaP Trans Dihydrodiols

Substrate	4,5-dihydrodiol Conformation[a,b]	7,8-dihydrodiol Conformation[a,b]	9,10-dihydrodiol Conformation[a,b]
6-MBaP	AA	AA	AA
BaP	EE	EE	AA

a AA is the abbreviation for quasi-diaxial.
b EE is the abbreviation for quasi-diequatorial.

ities of the metabolic enzymes towards the metabolism of these two substrates.

Rate of metabolite formation. Either [^{3}H]6-MBaP or [^{14}C]BaP was incubated with 3-MC-, PB-, or control rat liver microsomes as described in "Materials and Methods." All microsomal suspensions were frozen until use, however metabolism studies were performed within two weeks after microsomes were prepared since certain forms of cytochrome P-450 are known to be less stable than others over time under certain storage conditions (155). For purposes of comparison, the same preparation of each type of microsome was used to study the metabolism of [^{3}H]6-MBaP or [^{14}C]BaP.

The rates of BaP and 6-MBaP metabolite formation (expressed in pmoles of product per nmol cytochrome P-450 per minute) with each of the three microsomal preparations are shown in Tables 2 and 3. Each value shown represents the mean of two separate incubations. The rates of formation of all the metabolites listed in Tables 2 and 3 were linear with respect to time and the amounts of microsomal protein used.

HPLC and quantitative analysis of the 6-MBaP zero-time and 10-minute incubation blanks prepared with heat killed microsomes revealed a radioactive peak with the same retention time as the 6-OHMBaP metabolite of 6-MBaP (see Figs. 4 and 5). Although this peak represented only about 10% of the rate of formation of 6-OHMBaP when active PB- or control microsomes were used, it was found to be about 50% of the rate of 6-OHMBaP formation when active 3-MC-microsomes were incorporated in the incubation mixture. When the blank values were subtracted from the values obtained with active microsomal preparations, the values for the specific activity of 6-OHMBaP formation with 3-MC-, PB-, or control

TABLE 2

Metabolism of [^{3}H]6-MBaP by rat liver microsomes

Eighty nmol of [^{3}H]6-MBaP (specific activity, 62 mCi/mmol) were incubated with microsomes obtained from untreated rats and rats pretreated with PB or 3-MC, as described in "Materials and Methods." After incubation, 6-MBaP and its metabolites were prepared and analyzed by HPLC.

	Specific Activity[a] (pmol of product/nmol cytochrome P-450/min) Microsomes		
Metabolite	3-MC	PB	Control
4,5-diol	86 [5][b]	55 [29]	18 [15]
7,8-diol	172 [10]	5 [3]	8 [7]
9,10-diol	121 [7]	4 [2]	6 [5]
1-OH 6-OHMBaP	103 [6]	3 [2]	<0.5 [0]
3-OH 6-OHMaP	32 [2]	4 [2]	<0.5 [0]
6-OHMBaP	48 [2]	58 [31]	41 [34]
12-OH 6-MBaP	ND[c]	30 [16]	22 [18]
OH 6-MBaP Peak I[d]	ND	16 [8]	ND
OH 6-MBaP Peak II[e]	1203 [68]	14 [7]	26 [21]
Total	1765	189	122
% substrate metabolized	6	3	1.2

a Each value represents the mean of two separate incubations and is based on the total radioactivity in identified metabolite fractions.
b Numbers in brackets indicate percentage of all metabolites.
c ND, not detected.
d Tentatively identified by UV absorption spectral analysis as a monohydroxylated metabolite of 6-MBaP. See text for discussion.
e Mixture of monohydroxylated metabolites of 6-MBaP. One component has been tentatively identified as 1-OH 6-MBaP. Other components are thought to be 3-OH and 9-OH 6-MBaP.

TABLE 3

Metabolism of [^{14}C]BaP by rat liver microsomes

Eighty nmol of [^{14}C]BaP (specific activity, 26 mCi/mmol) were incubated with microsomes obtained from untreated rats and rats pretreated with PB or 3-MC, as described in "Materials and Methods". After incubation, BaP and its metabolites were prepared and analyzed by HPLC.

	Specific Activity[a] (pmol of product/nmol cytochrome P-450/min) Microsomes		
Metabolite	3-MC	PB	Control
4,5-diol	315 [6][b]	244 [24]	149 [11]
7,8-diol	680 [13]	30 [3]	45 [3]
9,10-diol	865 [17]	101 [10]	150 [12]
1,6-quinone	339 [7]	111 [11]	132 [10]
3,6-quinone	383 [7]	125 [13]	155 [12]
6,12-quinone	184 [4]	77 [8]	106 [8]
9-OH BaP	230 [5]	20 [2]	27 [2]
7-OH BaP	51 [1]	6 [1]	8 [1]
1-OH BaP	593 [11]	124 [12]	206 [16]
3-OH BaP	1502 [29]	157 [16]	321 [25]
Total	5142	995	1299
% substrate metabolized	11	8	4

a Each value represents the mean of two separate incubations and is based on the total radioactivity in identified metabolite fractions.

b Numbers in brackets indicate percentage of all metabolites.

microsomes were found to be those shown in Table 2. Similar analysis of zero-time and 10-minute BaP incubation blanks indicated a small radioactive peak with the same retention time as the 9-phenol metabolite of BaP. The peak represented up to 20% of the rate of formation of the metabolite depending on the type of microsome used. The specific activities for 9-OH BaP formation after subtraction of the blank value are shown in Table 3.

It appears (Tables 2 and 3) that the presence of the six methyl group resulted in a dramatic decrease in the overall rate of 6-MBaP metabolite formation compared with that of BaP metabolite formation by about 66, 81, or 91% with 3-MC-, PB-, and control microsomes respectively. Under identical incubation conditions, a lower percentage of 6-MBaP was metabolized than was BaP with any microsmal preparation.

A four-fold induction was seen in the overall catalytic activity of 3-MC-microsomes compared to control microsomes when BaP was metabolized (Tables 2 and 3). However, a 14.5-fold induction over control levels due to 3-MC-pretreatment was observed when 6-MBaP was the substrate. PB-pretreatment also induced the rate of formation of 6-MBaP metabolites 1.6 times; however, a slight decline in activity compared to control was observed when BaP was metabolized by PB-microsomes.

Comparison of BaP metabolism to total diol, quinone, and phenol metabolites and of 6-MBaP to total diol, total phenol, and total metabolites with a 6-hydroxymethyl moiety shows a similar relative distribution of metabolites in these categories with 3-MC-, PB-, and control microsomes (Table 4). Phenols of the parent hydrocarbon were the predominant metabolites formed with 3-MC- and control microsomes; however, slightly higher rates of formation were shown for 6-MBaP and BaP diols,

TABLE 4

Comparison of the Metabolism of [^{3}H]6-MBaP and [^{14}C]BaP to Particular Types of Metabolites

Substrate and Metabolite Type	Specific Activity[a] (pmol product/nmol cytochrome P-450/minute) Microsomes		
	3-MC	PB	Control
BaP			
Total diols	1860 (36)[b]	375 (37)	344 (26)
Total quinones	906 (18)	313 (32)	393 (30)
Total phenols	2376 (46)	307 (31)	562 (44)
6-MBaP			
Total diols	379 (22)	64 (34)	32 (27)
Total[c] metabolites with 6-hydroxy-methyl moiety	183 (10)	65 (35)	42 (34)
Total 6-MBaP phenols	1203 (68)	60 (31)	48 (39)

a Total rate of formation of metabolites in each category.
b Numbers in parenthesis indicate total percentage of all metabolites.
c Taken as analogous to BaP quinone formation since the 6-position methyl group of 6-MBaP blocks quinone formation but is itself metabolized.

BaP quinones, and 6-OHMBaP and its derivatives when PB-microsomes were used.

No convincing evidence could be obtained by UV absorption spectral analysis in the present study to indicate that the 5-phenol is a metabolite of BaP, and it has not been reported elsewhere to be a metabolite of BaP when rat liver microsomes were used as the source of enzymes (see elution position of BaP 5-phenol standard shown in Fig. 3).

In performance of these quantitative studies, it was not possible to achieve a baseline separation of the 6-MBaP 7,8- and 9,10-diols due to the relatively large volume of organic solvent needed to dissolve 6-MBaP and its metabolites for HPLC analysis. Therefore, an aliquot of the same metabolite mixture used for HPLC analysis of the other 6-MBaP metabolites was prepared as described previously and the 6-MBaP diol metabolites were separated using isocratic conditions as described in "Materials and Methods." A chromatogram of the 6-MBaP 4,5-, 7,8-, and 9,10-diols formed by 3-MC-microsomes appears in Fig. 21. The rates of formation of the diol metabolites isolated by this procedure are those given in Table 2.

Although the 4,5-, 7,8-, and 9,10-diols are relatively major metabolites of BaP when 3-MC-, PB-, and control microsomes are used and of 6-MBaP when 3-MC-microsomes are used, metabolism of 6-MBaP by PB- or control microsomes results in barely detectable levels of 7,8- and 9,10-diols although the 6-MBaP 4,5-diol is formed in more substantial amounts with these microsomes (Tables 2 and 3).

The ratios of the rates of BaP and 6-MBaP diol metabolite formation by the three microsomal preparations are given in Table 5. The extent of BaP 7,8-diol formation with 3-MC-, PB-, and control microsomes

Fig. 21. Separation of 6-MBaP dihydrodiol metabolites obtained when a mixture of [^{3}H]6-MBaP metabolites was applied to a Vydec C_{18} column and eluted with 50% methanol in water at a flow rate of 1 ml/minute. These conditions were used to quantify the 6-MBaP dihydrodiols. See text for discussion.

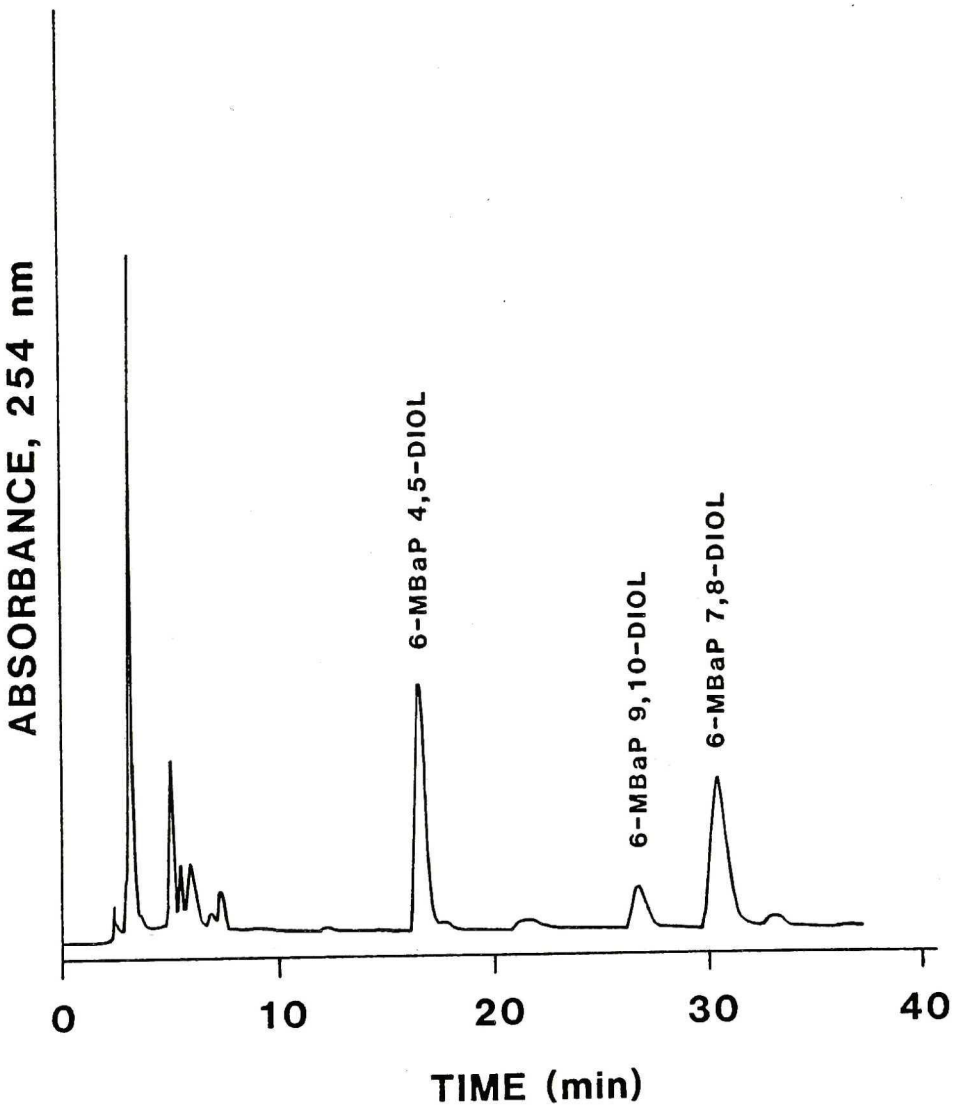

ABSORBANCE, 254 nm
6-MBaP 4,5-DIOL
6-MBaP 9,10-DIOL
6-MBaP 7,8-DIOL
0
10
20
30
40
TIME (min)

TABLE 5

Ratios of the Rates of BaP and 6-MBaP Dihydrodiol Formation

Microsome	BaP Dihydrodiol/6-MBaP Dihydrodiol[a]		
	Dihydrodiol Metabolite		
	4,5-diol	7,8-diol	9,10-diol
Control	8.3	5.6	25
PB	4.4	6	25.3
3-MC	3.7	4	7.2

a Ratio of specific activities.

respectively was 4, 6, and 5.6 times higher than that of the 6-MBaP 7,8-diol. Similar ratios were obtained when the BaP and 6-MBaP 4,5-diols were compared. Far greater differences in the extent of 6-MBaP 9,10-diol formation with PB- and control microsomes were observed.

Positional selectivity of microsomal enzymes. Differences in regioselectivity towards the metabolism of both 6-MBaP and BaP were noted in many instances with the three microsomal preparations used. 3-MC-microsomes favored formation of the BaP and 6-MBaP 9,10- and 7,8-diols, while PB- and control microsomes favored the formation of the 4,5-diols of both substrates (Tables 2 and 3).

12-Hydroxy 6-MBaP and OH 6-MBaP Peak I which were detected as metabolites of 6-MBaP were not found to be metabolites of BaP. Differences in positional selectivity shown by the various microsomes towards the formation of the BaP 9- and 3-phenols were observed in this study (as they had been by previous investigators). Since the BaP 1-phenol was separable from the other BaP phenol metabolites by the Vydec HPLC column, it was also possible to note differences in the reigoselectivities of the three microsomal preparations towards the formation of this phenol (Tables 2 and 3).

6-OHMBaP, a minor metabolite of 6-MBaP when 3-MC-microsomes were used was the predominant single metabolite formed by PB- and control microsomes (Table 2). Although significant amounts of the 6-OHMBaP 1- and 3-phenols were obtained with 3-MC-microsomes, their formation was virtually absent when PB- and control microsomes were used.

Stereoselectivity of Microsomal Enzymes

The spectra obtained by circular dichroism (CD) analysis of

purified 6-MBaP trans-4,5-, 7,8-, and 9,10-diol metabolites formed by 3-MC-microsomes appear in Figs. 22-24 along with the spectra of the corresponding BaP diol metabolites. Spectra of individual diol metabolites of 6-MBaP or BaP formed by PB- or control microsomes were virtually identical in shape and sign to the spectra recorded for a particular 6-MBaP or BaP diol obtained by the metabolism of either substrate with 3-MC-microsomes. A comparison of the spectral properties of the BaP and 6-MBaP trans diols appears in Table 6. These data indicate that the 6-MBaP diols metabolically formed by the various microsomal preparations are all optically active. Therefore, like BaP, 6-MBaP is stereoselectively metabolized to trans-4,5-, 7,8-, and 9,10-diol metabolites whose predominant enantiomers are characterized by strong (-) Cotton effects (for values and wavelengths see Table 6). The optical purity of the 6-MBaP diol metabolites and the sign of optical rotation of the predominant enantiomer formed could not be determined by these procedures. However, by comparing the CD data obtained in this study for each 6-MBaP diol metabolite with data recently reported in two other studies, it was possible to definitively establish the absolute configurations of the predominant enantiomers of the metabolically formed 6-MBaP 4,5-, 7,8-, and 9,10-diols (see "Discussion" section).

The six-methyl group induces conformational changes in the 6-MBaP 4,5- and 7,8-diol metabolites which are formed peri to it. These conformational effects resulted in altered CD spectra for these two diols compared to the spectra recorded for the corresponding BaP diols (Figs. 22 and 24). The major Cotton effect for the 6-MBaP 4,5-diol (in AA conformation) formed by 3-MC-microsomes was recorded at 236 nm while the major Cotton effect for the BaP 4,5-diol (in EE conformation) formed

Fig. 22. Circular dichroism spectra of the 6-MBaP and BaP 4,5-dihydrodiol metabolites formed by 3-MC-microsomes. Ellipticity, recorded in millidegrees (m°) on the Y-axis, is shown in two scales; scale A for the 6-MBaP trans-4,5-dihydrodiol (solid line), and scale B for the BaP trans-4,5-dihydrodiol (dashed line). The ellipticity for the major Cotton effect of each diol at the indicated wavelength is also labelled. The [4R,5R] absolute stereochemistry of the predominant enantiomer of each diol metabolite is indicated by the structures shown.

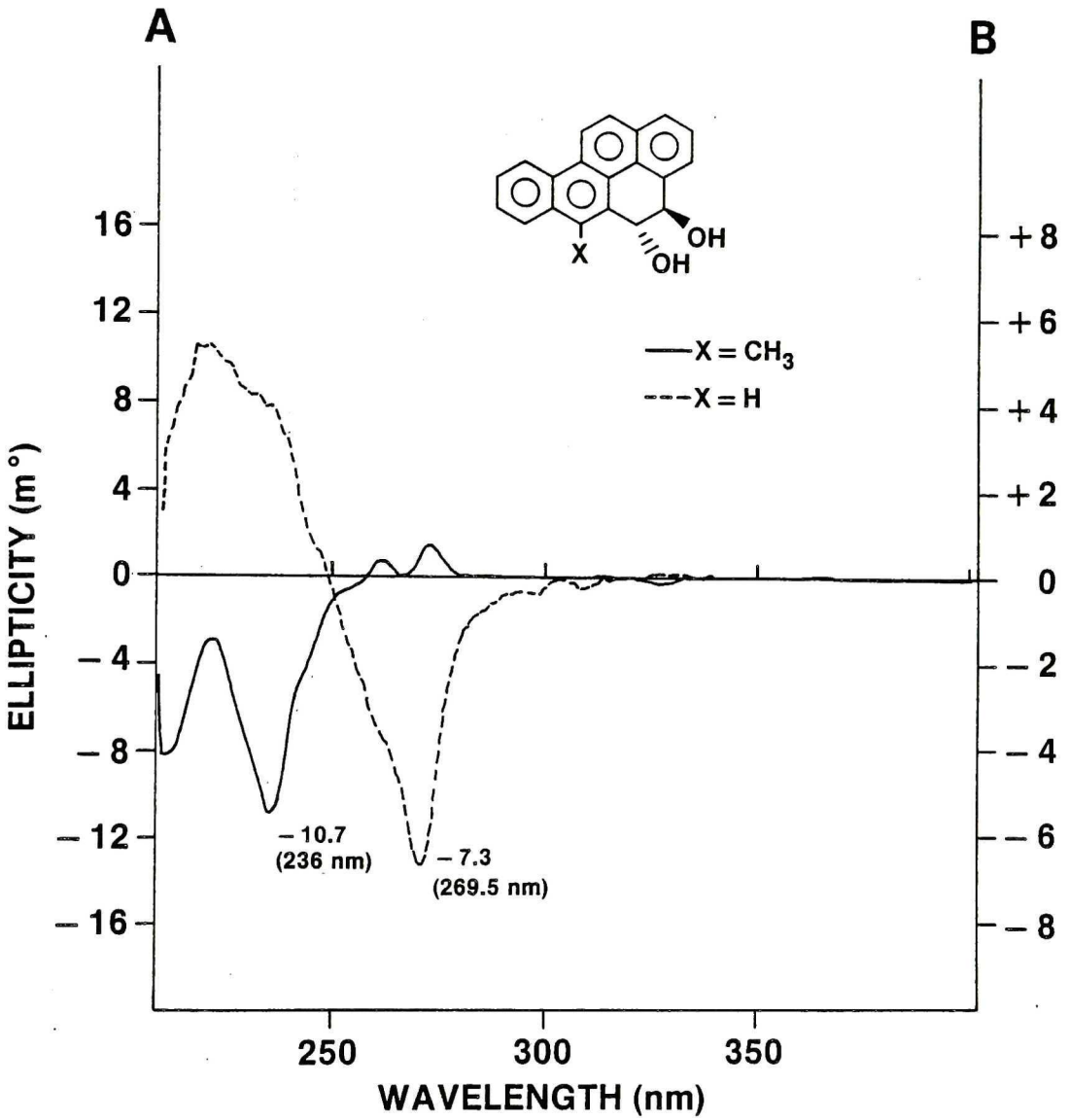

A
B
16
12
8
4
0
– 4
– 8
– 12
– 16
+ 8
+ 6
+ 4
+ 2
0
– 2
– 4
– 6
– 8
ELLIPTICITY (m°)
X
OH
OH
—X = CH3
---X = H
– 10.7
(236 nm)
– 7.3
(269.5 nm)
250
300
350
WAVELENGTH (nm)

Fig. 23. Circular dichroism spectra of the 6-MBaP and BaP 9,10-dihydrodiol metabolites formed by 3 MC-microsomes. Ellipticity, recorded in millidegrees (m°) on the Y-axis, is shown in two scales; scale A for the 6-MBaP trans-9,10-dihydrodiol (solid line), and scale B for the BaP trans-9,10-dihydrodiol (dashed line). The ellipticity for the major Cotton effect of each diol at the indicated wavelength is also labelled. The [9R,10R] absolute stereochemistry of the predominant enantiomer of each diol metabolite is indicated by the structures shown.

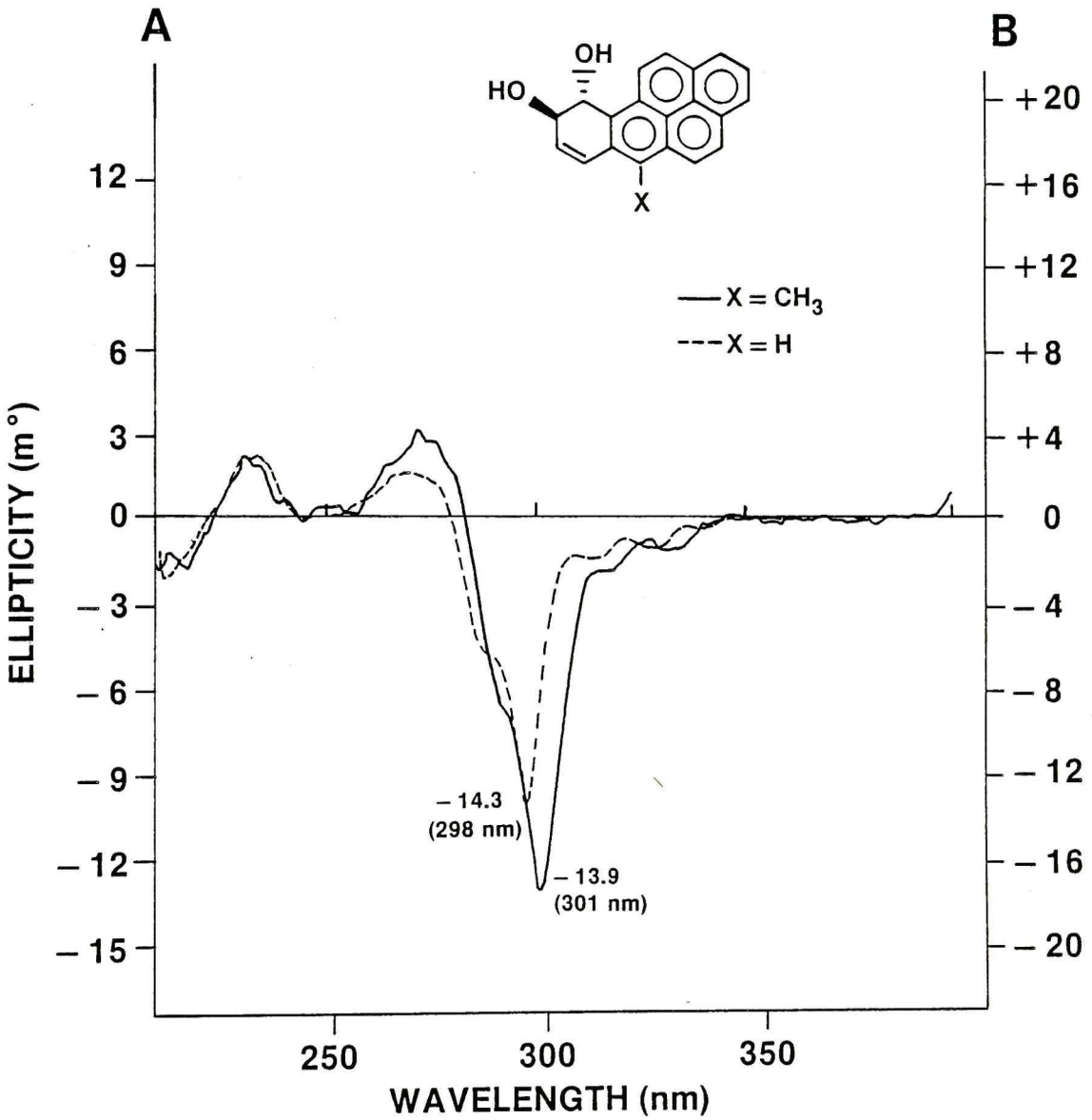

A
B
OH
HO
X
X = CH3
X = H
12
9
6
3
0
– 3
– 6
– 9
– 12
– 15
+20
+16
+12
+8
+4
0
– 4
– 8
– 12
– 16
– 20
ELLIPTICITY (m°)
– 14.3
(298 nm)
– 13.9
(301 nm)
250
300
350
WAVELENGTH (nm)

Fig. 24. Circular dichroism spectra of the 6-MBaP and BaP 7,8-dihydrodiol metabolites formed by 3-MC-microsomes. Ellipticity, recorded in millidegrees (m°) on the Y-axis, is shown in two scales; scale A for the 6-MBaP trans-7,8-dihydrodiol (solid line), and scale B for the BaP trans-7,8-dihydrodiol (dashed line). The ellipticity for the major Cotton effect of each diol at the indicated wavelength is also labelled. The [7R,8R] absolute stereochemistry of the predominant enantiomer of each diol metabolite is indicated by the structures shown.

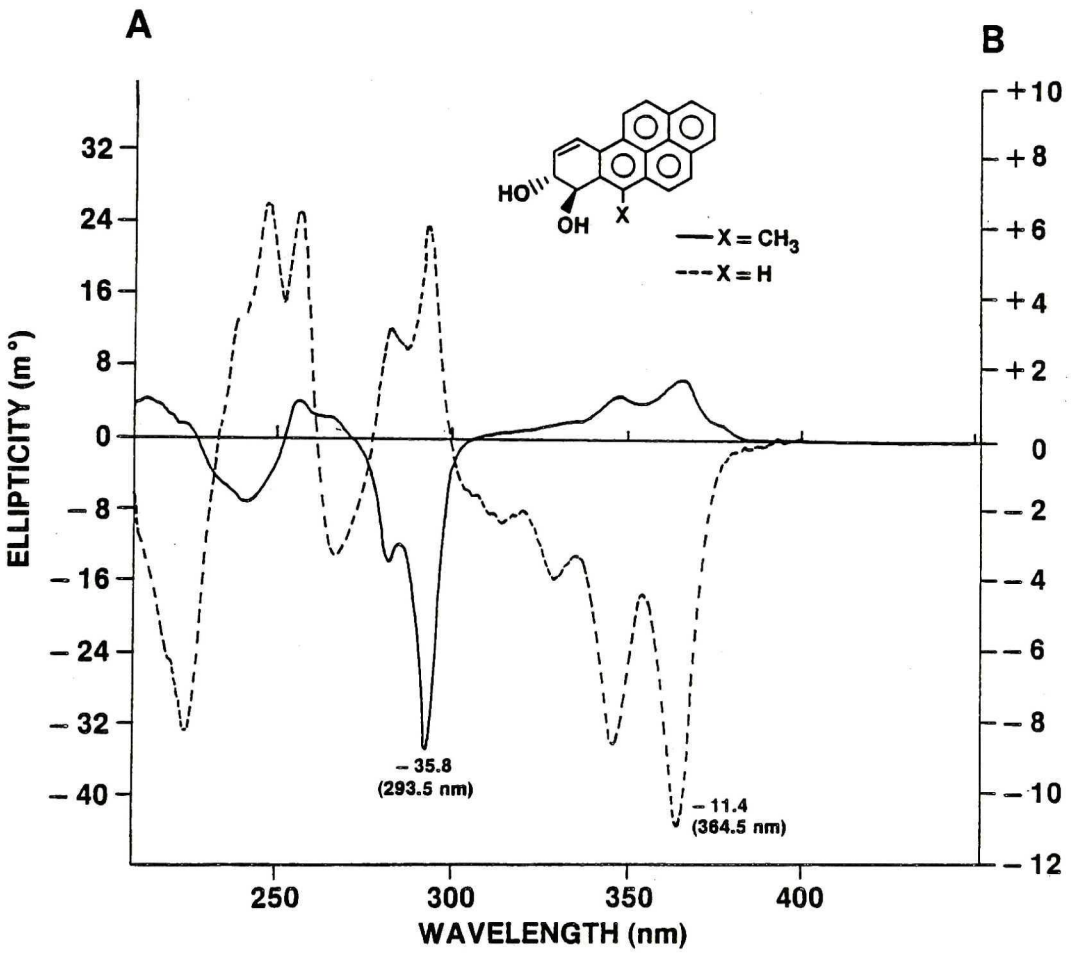

A
B
ELLIPTICITY (m°)
32
24
16
8
0
– 8
– 16
– 24
– 32
– 40
+10
+ 8
+ 6
+ 4
+ 2
0
– 2
– 4
– 6
– 8
– 10
– 12
HO
OH
X
X = CH3
X = H
– 35.8
(293.5 nm)
– 11.4
(364.5 nm)
250
300
350
400
WAVELENGTH (nm)

TABLE 6
CD spectral properties of dihydrodiols formed from the metabolism of 6-MBaP and BaP by liver microsomes 3-MC- and PB-pretreated rats and rats which received no pretreatment.[a]

Metabolites	Microsomes	ψ (λ)[b]	[θ] x 10^{-4} (λ)[c]
BaP-trans-4,5-dihydrodiol	3-MC	-7.30 (270 nm)	-6.52
6-MBaP-trans-4,5-dihydrodiol	3-MC	-10.70 (236 nm)	-9.56
6-MBaP-trans-4,5-dihydrodiol	PB	-12.70 (237 nm)	-11.35
6-MBaP-trans-4,5-dihydrodiol	Control	-12.00 (237 nm)	-10.70
BaP-trans-7,8-dihydrodiol	3-MC	-11.40 (365 nm)	-5.76
6-MBaP-trans-7,8-dihydrodiol	3-MC	-35.80 (294 nm)	-18.09
BaP-trans-9,10-dihydrodiol	3-MC	-14.30 (298 nm)	-9.99
6-MBaP-trans-9,10-dihydrodiol	3-MC	-13.90 (301 nm)	-9.72

a Spectra were recorded for samples dissolved in methanol.
b Ellipticity in millidegrees at indicated wavelengths.
c The extinction coefficients (in M^{-1} cm^{-1}) of the trans dihydrodiols of BaP (156) and 6-MBaP at absorption maxima used for calculating [θ] are: 89 365 (272 nm for BaP 4,5-dihydrodiol and 275 nm for 6-MBaP 4,5-dihydrodiol); 50 530 (366 nm for BaP 7,8-dihydrodiol and 369 nm for 6-MBaP 7,8-dihydrodiol); 69 930 (278 nm for BaP 9,10-dihydrodiol and 283 nm for 6-MBaP 9,10-dihydrodiol).

by 3-MC-microsomes was recorded at 269.5 nm. Even more drastic spectral alterations were noted for the 6-MBaP 7,8-diol (in AA conformation) when its spectrum was compared with that of the BaP 7,8-diol (in EE conformation). These effects are not due to differences in the absolute configurations of the two 7,8-diol metabolites but rather to a change in the skew sense of the terminal ring double bond relative to the remainder of the π aromatic system (158). In contrast, the Cotton effects of the 6-MBaP and BaP 9,10-diol metabolites (Fig. 23) are virtually identical. Both of these metabolites are in the AA conformation due to steric hindrance in the bay region.

Metabolism of 6-OHMBaP by Rat Liver Microsomes

6-MBaP is metabolized to 6-OHMBaP and the 1- and 3-phenols of 6-OHMBaP. There are two possible ways by which these phenols could form metabolically: 1. 6-MBaP could first be metabolized to 6-OHMBaP which could then be further metabolized to form the 1- or 3-phenol metabolite of 6-OHMBaP; 2. 6-MBaP could first be metabolized to 1- and 3-OH 6-MBaP. The 6-methyl group of either phenol could then be further metabolized to form the 6-OHMBaP derivative. Since radiolabelled 6-OHMBaP was available, the first possibility was explored. There were additional reasons for examining the microsomal metabolism of 6-OHMBaP. 6-OHMBaP is purported to be involved in an activation pathway of 6-MBaP via sulfate ester formation (52). However, although the carcinogenic and mutagenic activity of 6-OHMBaP have been determined in various systems (52,96,116,128,141-143), the *in vitro* metabolism of this compound by rat liver microsomes has not been explored.

Identification of 6-OHMBaP Metabolites

Incubations of 6-OHMBaP with rat liver microsomes and separation and identification of 6-OHMBaP metabolites were carried out using procedures like those used in the 6-MBaP metabolism studies.

The reversed-phase HPLC chromatogram of 6-OHMBaP metabolites formed by 3-MC-microsomes appears in Fig. 25. The identities of the 6-OHMBaP metabolites are (in order of increasing retention time and decreasing polarity): 6-OHMBaP 9,10-dihydrodiol (6-OHMBaP 9,10-diol), 6-OHMBaP 4,5-dihydrodiol (6-OHMBaP 4,5-diol), 6-OHMBaP 7,8-dihydrodiol (6-OHMBaP 7,8-diol), 1-hydroxy-6-OHMBaP (1-OH 6-OHMBaP), 3-hydroxy-6-OHMBaP (3-OH 6-OHMBaP), followed by the 6-OHMBaP substrate peak. An arrow indicates the position of elution of a BaP 6-aldehyde standard. This compound was not found to be a metabolite of 6-OHMBaP with 3-MC, PB-, or control microsomes. Peak A, Peak B, and Peak C are discussed below.

The elution order of the 6-OHMBaP 4,5- and 9,10-diols is like that of the corresponding BaP diols rather than that of the analogous 6-MBaP diols. Hydrogen bonding between the 6-hydroxymethyl group of 6-OHMBaP and the hydroxyl group located at position 5 of the 6-OHMBaP 4,5-diol may occur and alter the polarity of this diol with respect to the 6-OHMBaP 9,10-diol.

The radiogram shown in Fig. 26 was obtained after HPLC separation and quantitative analysis of the metabolites of [^{3}H]6-OHMBaP formed by 3-MC-microsomes using procedures described in "Materials and Methods." Chromatographic conditions were identical to those used to separate the metabolites formed from unlabelled substrate (Fig. 25). Radiograms obtained upon analyses of [^{3}H]6-OHMBaP metabolism by PB- or control microsomes were similar to that shown for 3-MC-microsomes and so are not

Fig. 25. Reversed-phase HPLC profile of 6-OHMBaP metabolites formed by 3-MC-microsomes. The arrow indicates the elution position of a BaP 6-aldehyde standard which was added to the metabolite mixture prior to chromatography. See text for discussion. Metabolites were eluted from a Vydec C_{18} HPLC column with a 35-minute linear gradient of 50 to 90% methanol in water at a flow rate of 1 ml/minute.

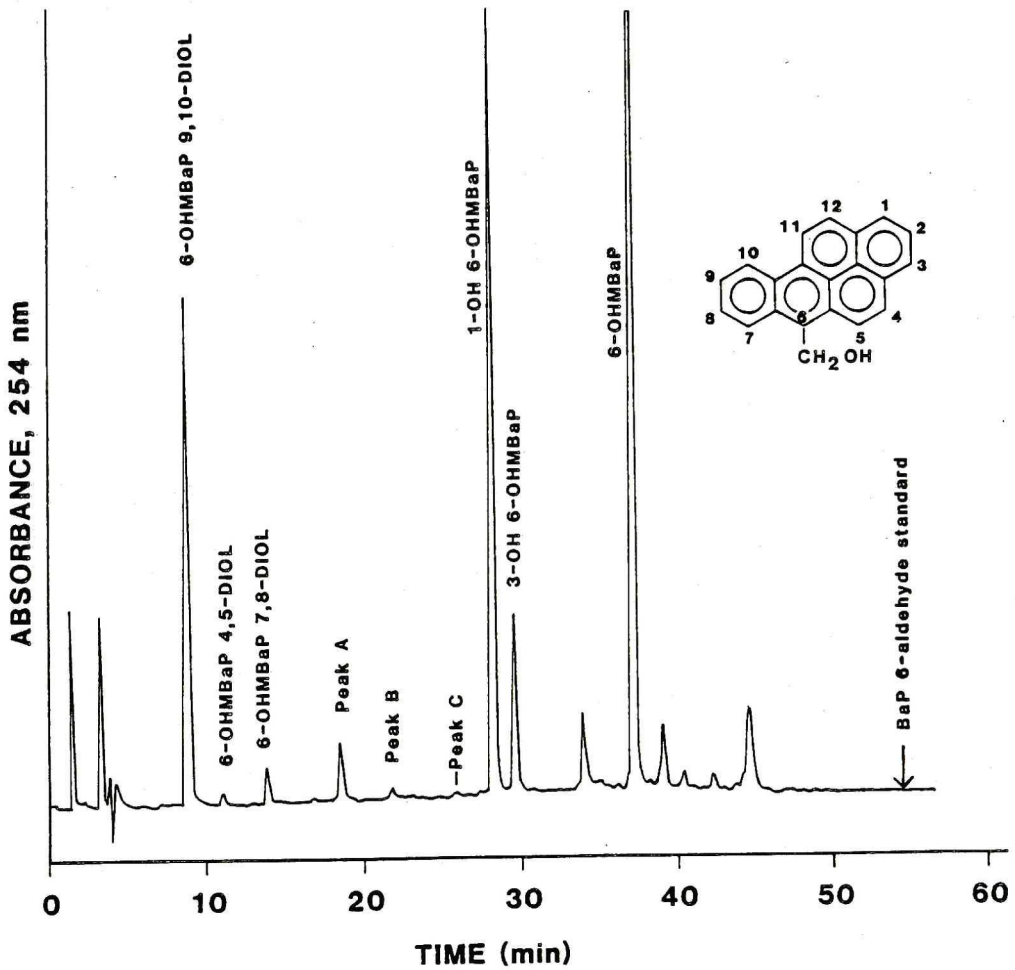
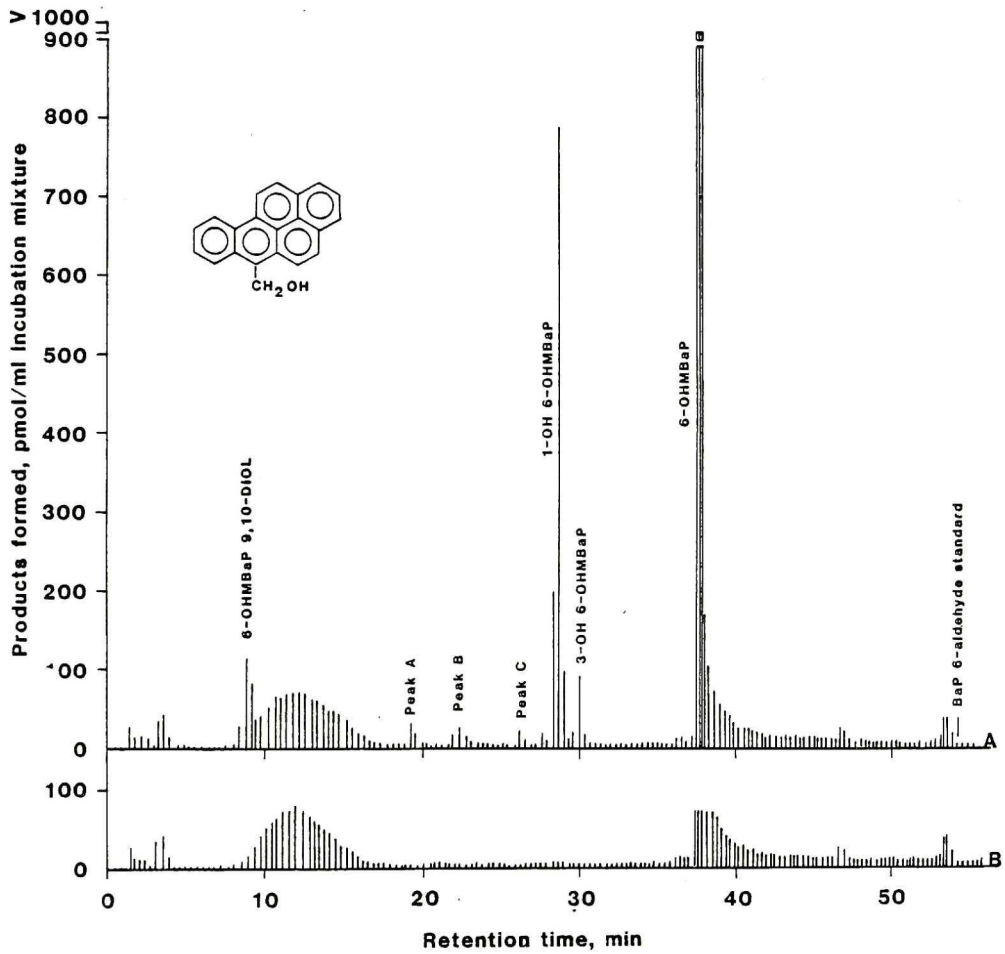

ABSORBANCE, 254 nm
6-OHMBaP 9,10-DIOL
6-OHMBaP 4,5-DIOL
6-OHMBaP 7,8-DIOL
Peak A
Peak B
Peak C
1-OH 6-OHMBaP
3-OH 6-OHMBaP
6-OHMBaP
BaP 6-aldehyde standard
12
1
11
2
10
3
9
8
4
7
6
5
CH2 OH
0
10
20
30
40
50
60
TIME (min)

Fig. 26. Axis A. The profile of metabolites formed from the metabolism of [^{3}H]6-OHMBaP by 3-MC-microsomes after correction for background radioactivity. Axis B. Corresponding zero-time incubation blank after correction for background radioactivity. Similar results were obtained upon analysis of the 5-minute incubation blank. Relevant procedures were described in "Materials and Methods." Reversed-phase chromatographic conditions were identical to those described in Fig. 25.


Products formed, pmol/ml incubation mixture
1000
900
800
700
600
500
400
300
200
100
0
100
0
0
10
20
30
40
50
Retention time, min
CH2OH
6-OHMBaP 9,10-DIOL
Peak A
Peak B
Peak C
1-OH 6-OHMBaP
3-OH 6-OHMBaP
6-OHMBaP
BaP 6-aldehyde standard
A
B

presented.

Metabolite peaks indicated in the radiogram (Fig. 26) as the 6-OHMBaP 9,10-diol and the 6-OHMBaP 1- and 3-phenols clearly correspond to particular peaks in the chromatogram shown in Fig. 25, as do the peaks labelled Peak A, Peak B, and Peak C. However, HPLC and quantitative analyses of the [^{3}H]6-OHMBaP zero-time and 5- and 10-minute incubation blanks prepared with heat killed 3-MC-, PB-, and control microsomes revealed a broad peak of radioactivity which eluted in the same region as the 6-OHMBaP 4,5- and 7,8-diol UV marker metabolites. Similar amounts of this radioactive impurity were found upon analysis of incubation mixtures containing active 3-MC, PB-, and control microsomes. Therefore, it is doubtful that large rates of formation of the 6-OHMBaP 4,5- and 7,8-diols occurred; however, small rates of formation of these two diol metabolites could have been obscured by the impurity and hence, could not be quantified. The impurity was present only in the radiolabelled 6-OHMBaP and not in the unlabelled 6-OHMBaP used to perform the qualitative metabolism studies. Thus, the 4,5- and 7,8-diol region is not obscured in the chromatogram shown in Fig. 25.

UV absorption spectral analysis. Tentative identification of the 6-OHMBaP metabolites was provided by UV absorption spectral analysis. Absorption spectra of the 6-OHMBaP diols and those of the corresponding BaP and 6-MBaP diols were virtually identical and so are not shown. Like the 6-MBaP diols, the spectra of the 6-OHMBaP diols showed slight bathochromic shifts. The UV absorption spectra of the 6-OHMBaP 1- and 3-phenols were the same as those presented for these compounds as metabolites of 6-MBaP (Figs. 6 and 10). Spectra obtained for the three metabolites of 6-OHMBaP labelled Peak A, B, or C in the chromatogram

in Fig. 25 were not similar to any of the spectra available for BaP metabolites or standards. Peaks A, B, and C were found to be very minor metabolites of 6-OHMBaP when quantitative studies were performed (to be described later). The UV absorption spectra for Peak A obtained in methanol and in the presence of alkali are shown in Fig. 27. Upon the addition of NaOH, the bathochromic shift characteristic of phenolic aromatic compounds was evident, but nothing more specific than this can be said about the nature of Peak A based on the available information.

Mass spectral analysis. Mass spectral analysis of the metabolites of 6-OHMBaP tentatively identified as the 6-OHMBaP 4,5-, 7,8-, and 9,10-diols and the 6-OHMBaP 1- and 3-phenols confirmed these designations. Mass spectral data for the 6-OHMBaP 1- and 3-phenols were identical to that presented for these compounds as metabolites of 6-MBaP (Figs. 17 and 18). The mass spectra for the three 6-OHMBaP diols appears in Figs. 28-30. Their molecular weights were confirmed by the presence of mass ions at m/z 316. Characteristic ion fragments were also noted at m/z 298 (loss of water), 281 (loss of water and OH), 280 (loss of $2H_2O$), 269 (loss of OH and CH_2O), 252 (loss of two OH groups and CH_2O) and 239 (breakup of ring structure).

Attempts to characterize Peaks A, B, and C by mass spectral analysis were hampered by the lability of these compounds even in the presence of ascorbic acid. Attempts to make acetate derivatives of the three metabolites were also unsuccessful.

Quantification of 6-OHMBaP Metabolism by Rat Liver Microsomes

The metabolites of [^{3}H]6-OHMBaP formed by 3-MC-, PB-, and control rat liver microsomes were quantified using procedures already described. For the microsomal protein concentrations used, metabolite formation

Fig. 27. Ultraviolet-visible absorption spectra of a 6-OHMBaP metabolite labelled Peak A in Figs. 25 and 26 (in methanol, ———; in the presence of alkali,- - - - -).

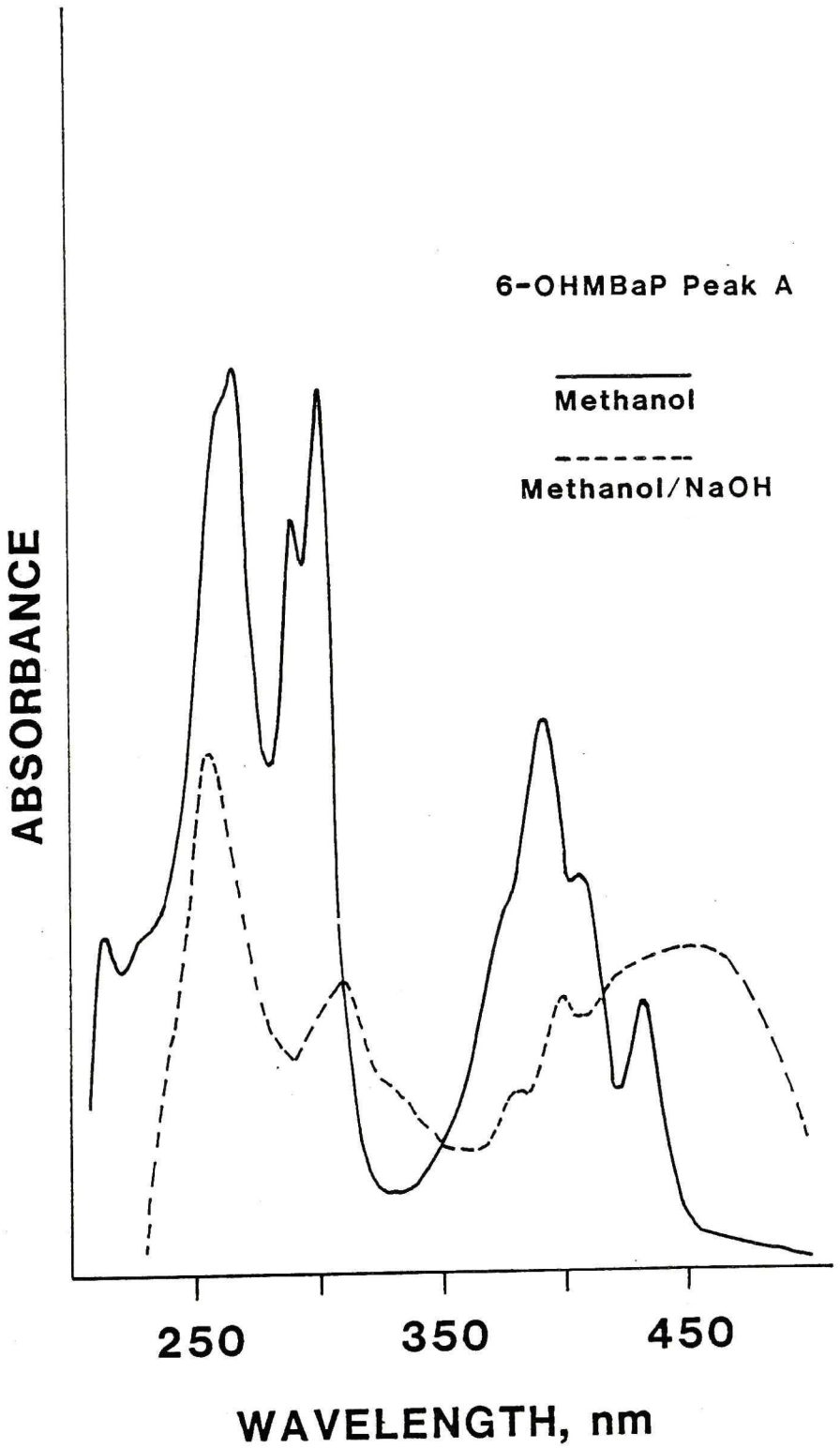

6-OHMBaP Peak A
Methanol
Methanol/NaOH
ABSORBANCE
250
350
450
WAVELENGTH, nm

Fig. 28. Mass spectrum of the 4,5-dihydrodiol metabolite of 6-OHMBaP. The mass ion is indicated as (M+) and characteristic ion fragments are labelled.

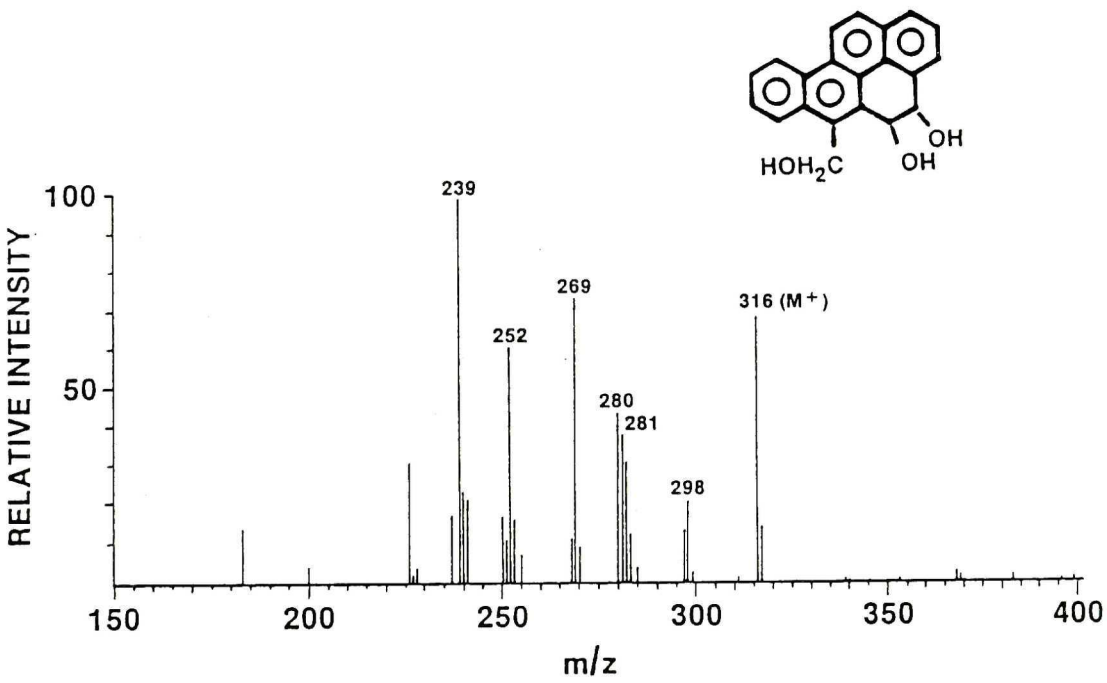

OH
HOH2C
OH
100
50
RELATIVE INTENSITY
239
269
316 (M+)
252
280
281
298
150
200
250
300
350
400
m/z

Fig. 29. Mass spectrum of the 7,8-dihydrodiol metabolite of 6-OHMBaP. The mass ion is indicated as (M+) and characteristic ion fragments are labelled.

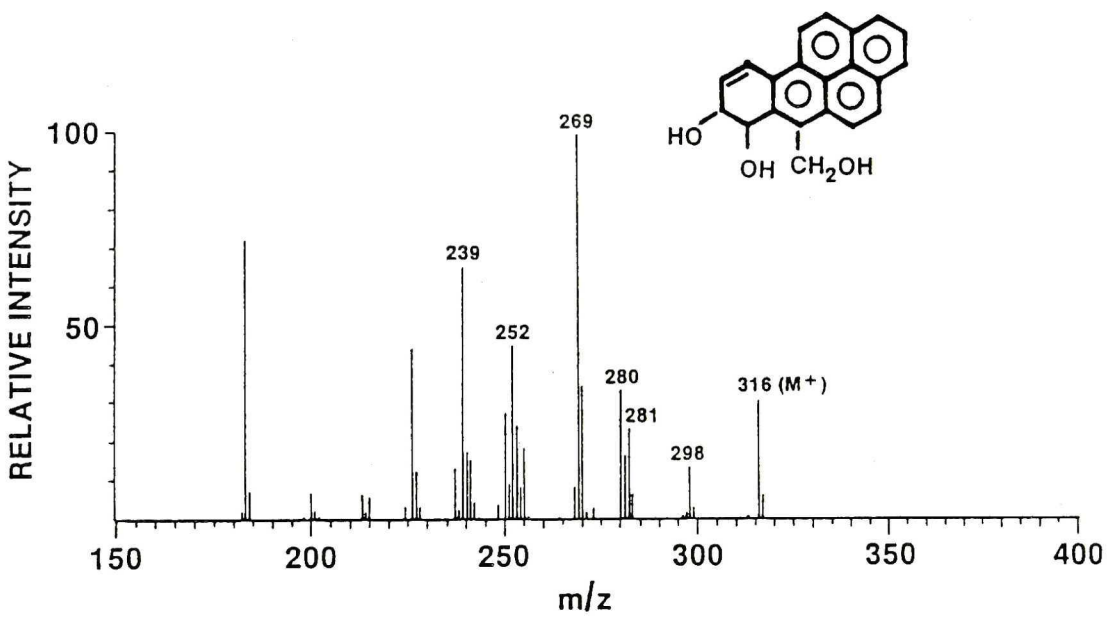
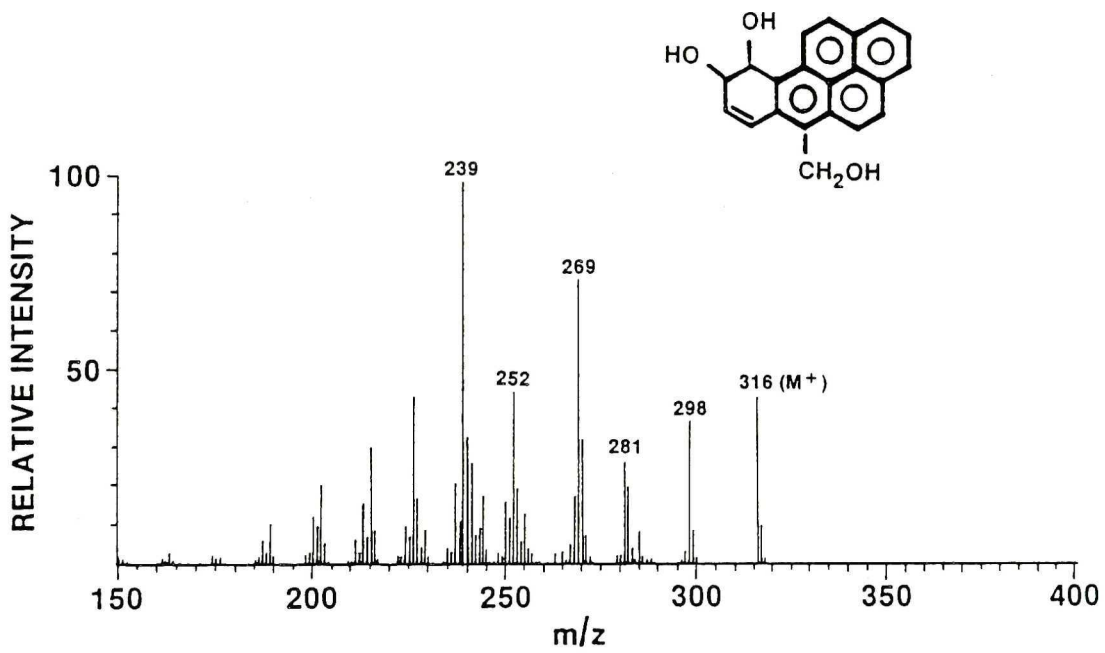

RELATIVE INTENSITY
100
50
150
200
250
300
350
400
m/z
269
239
252
280
281
298
316 (M+)
HO
OH
CH2OH

Fig. 30. Mass spectrum of the 9,10-dihydrodiol metabolite of 6-OHMBaP. The mass ion is indicated as (M+) and characteristic ion fragments are labelled.


OH
HO
CH2OH
100
50
RELATIVE INTENSITY
239
269
252
316 (M+)
298
281
150
200
250
300
350
400
m/z

was found to be linear for incubation time periods of 5 minutes for 3-MC-microsomes and 10 minutes for PB- and control microsomes. The microsomal enzyme preparations used to quantify 6-OHMBaP metabolism were prepared at different times than those used to quantify 6-MBaP and BaP metabolism (see "Materials and Methods" for characterization) and had been stored frozen at -81°F for a number of months before use. Therefore, compared to fresh microsomal preparations, their metabolic activity was somewhat lower although the exact reduction in activity is not known. PB- and control microsomal suspensions may be somewhat more labile than 3-MC-microsomes upon storage in the frozen condition (155). In addition, since the mechanism of formation of various types of metabolites (diols, phenols, etc.) is known to differ and more than one enzyme or one metabolic step may be involved, decreases in their formation due to enzyme activity loss may not be uniform. The data which follows are presented with these reservations in mind.

The rate of formation (expressed in picomoles of product per nmol cytochrome P-450 per minute) of the metabolites of 6-OHMBaP by 3-MC-, PB-, and control microsomes are given in Table 7. Conversion of substrate to the 6-OHMBaP 1- and 3-phenols accounted for the largest percentages of metabolites formed by all of the three microsome preparations. More selectivity was shown by PB- and control microsomes towards the formation of 3-OH 6-OHMBaP than by 3-MC-microsomes. Conversely, 3-MC-microsomes preferentially metabolized 6-OHMBaP at the 1-position to form 1-OH 6-OHMBaP. 6-OHMBaP diol formation by any microsome preparation was very low or absent except for the formation of the 6-OHMBaP 9,10-diol by 3-MC-microsomes. Metabolites labelled as Peaks A, B, and C accounted for 10% or less of the metabolites formed by

TABLE 7

Metabolism of [^{3}H]6-OHMBaP by rat liver microsomes

Eighty nmol of [^{3}H]6-OHMBaP (specific activity, 141 mCi/mmol) were incubated with microsomes obtained from untreated rats and rats pretreated with PB or 3-MC, as described in "Materials and Methods". After incubation, 6-OHMBaP and its metabolites were prepared and analyzed by HPLC.

	Specific Activity (pmol of product/nmol cytochrome P-450/min) Microsomes		
Metabolite	3-MC[a]	PB[b]	Control[b]
9,10-diol	204 [14][c]	1 [1]	4 [1]
4,5-diol	NQ[d]	NQ	NQ
7,8-diol	NQ	NQ	NQ
Peak A	51 [3]	ND[e]	ND
Peak B	32 [2]	ND	ND
Peak C	24 [2]	8 [8]	38 [10]
1-OH 6-OHMBaP	1086 [72]	27 [28]	161 [41]
3-OH 6-OHMBaP	112 [7]	62 [63]	188 [48]
Total	1509	98	391
% substrate metabolite	6.2	2.5	2.7

a Each value represents the mean of two separate incubations and is based on the total radioactivity in identified metabolite fractions.
b Value indicates total radioactivity in identified metabolite fractions based on one sample determination.
c Numbers in brackets indicate percentage of all metabolites.
d NQ, not quantified. Could not be quantified due to presence of impurities.
e ND, not detected.

3-MC-, PB-, or control microsomes.

DISCUSSION

Metabolic pathways involved in the *in vitro* microsomal metabolism of 6-MBaP to a number of primary and secondary metabolites have been established. These pathways are illustrated in Fig. 31. Steps thought to involve the mixed function oxidases or epoxide hydrolase are indicated.

In terms of general types of metabolites produced, the BaP and 6-MBaP metabolite profiles are similar in that these substrates are converted to diols and phenols. However, the six-methyl group blocks quinone formation and is itself metabolized to yield 6-OHMBaP and two 6-OHMBaP phenol metabolites not found as metabolites of BaP. The methyl group does not seem to shift metabolism to any particular part of the 6-MBaP molecule since the percentages of total metabolism at various regions of the 6-MBaP and BaP molecules are very similar when a particular type of microsomes is used as the source of metabolic enzymes.

Both BaP and 6-MBaP were metabolized to diol metabolites of the *trans* geometric configuration. Although metabolism of BaP and 6-MBaP by 3-MC-, PB-, or control microsomes favored the formation of certain diol metabolites, the rates of formation of all of the 6-MBaP diols compared to those of BaP were reduced by 72 to 96% depending on the diol metabolite compared and the microsomal preparation used. The reduction in 6-MBaP diol formation at positions 4,5 and 7,8 which are *peri* to the six-methyl substituent suggests that these sites may not be readily accessible to metabolic attack due to the bulky methyl substituent.

In vitro microsomal conversion of BaP to its ultimate carcinogen, the 7,8-diol 9,10-epoxide, proceeds via a *trans* 7,8-diol intermediate. If 6-MBaP is activated to tumorigenic species via a pathway that

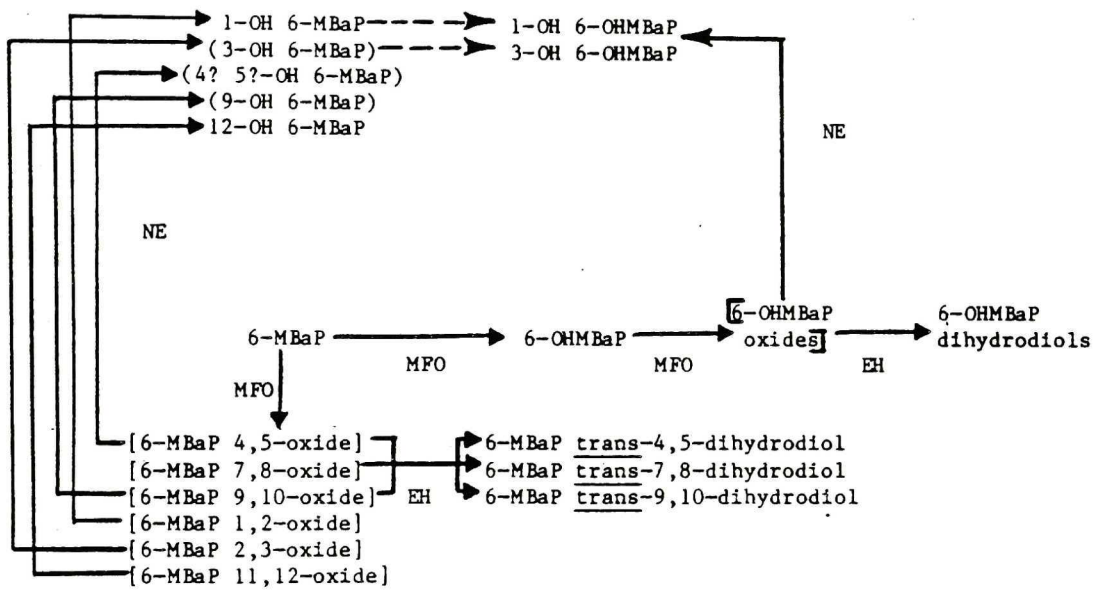


Fig. 31. In vitro metabolism of 6-MBaP by rat liver microsomes as elucidated in the present study. Metabolites appearing in brackets are presumed intermediates which were not isolated under the experimental conditions used. Metabolites appearing in parentheses are putative. Broken arrows represent possible pathways of metabolite formation. Mixed function oxidases are abbreviated by MFO, epoxide hydrolase is abbreviated by EH, and nonenzymatic rearrangement is abbreviated by NE.

involves a trans-7,8-diol metabolite intermediate, the lower amount of this diol formed relative to that formed of the BaP trans-7,8-diol might account, at least in part, for the lower tumorigenicity of 6-MBaP relative to that of BaP since comparatively less 6-MBaP 7,8-diol would be available for further metabolism to diol epoxides. The relatively low carcinogenicity of benz[a]anthracene is thought to stem, at least in part, from low levels of formation of the diol precursor of the bay region vicinal diol epoxide (77). Studies with benzo[e]pyrene (BeP), which like 6-MBaP but unlike BaP has its terminal benzo ring located in two bay regions, have suggested that the weak carcinogenicity of this compound coincides with low levels of metabolism of BeP to the BeP trans-9,10-diol which is the diol metabolite precursor of the bay region vicinal diol epoxide (159,160). The fluoro substituent of 12-fluoro-5-methylchrysene (12-F-5-MeC) is located peri to a terminal benzo ring. This substituent inhibited metabolism at the terminal ring, and the tumorigenicity of 12-F-5-MeC was weaker than its non-fluorinated counterpart (132,139). A known exception to the cases just cited involved a study in which the conversion of 6-fluorobenzo-[a]pyrene (6-FBaP) and BaP to 7,8-diol metabolites was compared. The six-fluoro substituent of 6-FBaP is peri to the terminal benzo ring of the molecule. Although amounts of 6-FBaP 7,8-diol higher than that of BaP 7,8-diol were formed, the 6-FBaP diol was found to possess lower biological activity than the BaP diol (158).

In the last two examples, the presence of a peri fluoro substituent resulted in the decreased biological activity of 6-FBaP and 12-F-5-MeC relative to their respective non-fluorinated counterparts. However, in the case of 6-FBaP, there was no concommitant decrease in

formation of the diol precursor of the bay region vicinal diol epoxide as there was with 12-F-5-MeC. Hence, the peri effect does not seem to be universally explicable on the basis of decreased formation of critical diol metabolites alone. There is another important aspect that must be considered in trying to understand the molecular basis(es) for the peri effect in general and in trying specifically to explain the differences in tumorigenicity observed for BaP and 6-MBaP. This aspect deals with the conformation of dihydrodiol metabolites which may be intermediates in the activation pathway of alkyl- or halogen-substituted PAH. The conformation preferentially adopted by the BaP trans-7,8-diol is quasi-diequatorial, while the conformational preference of the 6-MBaP trans-7,8-diol, which forms in relatively smaller amounts, is quasi-diaxial due to steric hindrance by the six-methyl group. Other trans diols of PAH with alkyl- or halogen-substituents located peri to the trans diol hydroxyl groups have also been found to preferentially adopt the AA conformation (for examples see 133,135,158, 161, 162). Although further metabolism of the 6-MBaP trans-7,8-diol to diol epoxides was not examined in this study, quasi-diaxial trans diols with adjacent double bonds in the bay region have been found to be poorly converted to bay region vicinal diol epoxides compared to their quasi-diequatorial counterparts (for examples see 114,135)*. Even if formed, diol epoxides with the diols in the AA conformation have been found to be weak mutagens and tumorigens (79,134,159,163-166).

BaP is stereospecifically metabolized by microsomal enzymes to (-)-trans-4,5-, 7,8-, and 9,10-diol metabolites. 6-MBaP was also

*The trans-9,10-diol of BeP is also an example of a quasi-diaxial trans diol with an adjacent double bond in a bay region (135,159).

found to be stereoselectively metabolized by 3-MC-, PB-, and control microsomes to optically active trans diol metabolites. The absolute configurations of the major enantiomers of the three trans BaP diols have all been determined to be [R,R] (167-169). Direct comparison of the CD spectra of the BaP trans-9,10-diol and the 6-MBaP trans-9,10-diol formed from 3-MC-microsomes revealed that both CD spectra are identical in shape and sign (except for a slight red shift of 4 nm shown by the 6-MBaP diol). Thus, it can be concluded that the predominant enantiomer of the enzymatically formed 6-MBaP trans-9,10-diol has the [9R,10R] absolute configuration. The hydroxyls of both diols lie in the bay region of their respective parent hydrocarbons, and due to steric crowding of the hydroxyls in the bay region, both diols have the AA conformation. Since the six-methyl group lies some distance away from the asymetric centers of the molecule, it apparently has no significant influence on the optical properties of the 6-MBaP trans-9,10-diol.

Because of substituent-induced conformational changes in the 6-MBaP trans-4,5- and 7,8-diols relative to the BaP trans-4,5- and 7,8-diols, direct comparison of 6-MBaP diol and BaP diol CD spectra to assign absolute configuration is not possible. However, when the CD data obtained in this study were compared with evidence presented in two recent published reports, it was possible to establish the absolute configurations of the predominant enantiomers of the 6-MBaP trans-7,8- and 4,5-diol metabolites.

In a study by Buhler et al. (158), the absolute configuration of the predominant enantiomer of the 6-FBaP trans-7,8-diol formed stereoselectively from 6-FBaP by liver microsomes prepared from rats pretreated with 3-MC, was deduced by the exciton chirality method to be

[7R,8R]. The hydroxyl groups of the 6-FBaP trans-7,8-diol are located peri to the 6-fluoro substituent (analogous to the 6-methyl group of 6-MBaP) and like the hydroxyl groups of the 6-MBaP trans-7,8-diol, preferentially adopt the AA conformation. A comparison of the CD spectrum recorded for the 6-FBaP trans-7,8-diol (presented in reference 158) with the CD spectrum of the 6-MBaP trans-7,8-diol metabolite (Fig. 24) showed the two spectra to be identical in shape and sign except for a slight bathochromic shift of 2 nm in the 6-MBaP diol spectrum. It can, therefore, be deduced that the absolute configuration of the predominant enantiomer of the 6-MBaP trans-7,8-diol is also [7R,8R].

It was possible to make the same deduction based on the results obtained by a study conducted by Yang et al. (162) which was carried out in order to determine the absolute configuration of the predominant enantiomer of the 6-bromobenzo[a]pyrene trans-7,8-diol formed stereoselectively from 6-bromobenzo[a]pyrene (6-BrBaP) by liver microsomes prepared from rats pretreated with 3-MC. Due to steric hindrance and electronic repulsion between the bromine substituent and the peri hydroxyl oxygen, the hydroxyl groups of this diol are forced to preferentially adopt the AA conformation. The CD spectrum of the 6-BrBaP trans-7,8-diol (illustrated in reference 162) is identical in shape and sign to that of the 6-MBaP trans-7,8-diol metabolite (with AA conformation) (Fig. 24) except for the slight red shift of 3.5 nm shown in the 6-BrBaP diol spectrum. When enzymatically formed 6-BrBaP trans-7,8-diol was converted by catalytic hydrogenolysis to the BaP 7,8,9,10-tetrahydro-trans-7,8-diol (with EE conformation), the Cotton effects appearing in the CD spectrum of this diol and the retention time of

the diol on a chiral HPLC column were identical to those of authentic BaP (+)-7,8,9,10-tetrahydro-trans-[7R,8R]-diol. Therefore, it was possible for the investigators to conclude that the absolute configuration of the major enantiomer of the 6-BrBaP trans-7,8-diol was [7R,8R]. Furthermore, it can be deduced that the absolute configuration of the major enantiomer of the 6-MBaP trans-7,8-diol is also [7R,8R].

In the same study (162), the absolute configuration of the predominant enantiomer of the 6-BrBaP trans-4,5-diol formed stereoselectively by 3-MC-microsomes was also determined. The CD spectrum of the 6-BrBaP trans-4,5-diol (shown in reference 162) is identical in shape and sign to that of the 6-MBaP trans-4,5-diol (Fig. 22) except for the bathochromic shift of 1 nm shown in the 6-BrBaP diol spectrum. Both diols have hydroxyl groups which preferentially adopt the AA confortion. Catalytic debromination of the enzymatically formed 6-BrBaP trans-4,5-diol resulted in the formation of BaP trans-4,5-diol (with EE conformation) which had a CD spectrum virtually identical to that of BaP (-)-trans-[4R,5R]-diol. Therefore, it was possible for the investigators to conclude that the absolute configuration of the major enantiomer of the 6-BrBaP trans-4,5-diol was [4R,5R]. Furthermore, it can be deduced that the absolute configuration of the major enantiomer of the 6-MBaP trans-4,5-diol is also [4R,5R].

Thus, 6-MBaP, like BaP is stereoselectively metabolized by rat liver microsomes to trans diol metabolites whose predominant enantiomers all have [R,R] absolute stereochemistry. Since the CD spectrum for a particular 6-MBaP trans diol metabolite formed by PB- or control microsomes had Cotton effects virtually identical in shape and sign to those found in the CD spectrum of the analogous 6-MBaP diol metabolite

formed by 3-MC-microsomes (as reported in "Results"), stereoselective formation of the [R,R] enantiomer occurred regardless of the type of microsomal preparation used in the present study. Similarly, other investigators have shown that the major enantiomers of the trans diol metabolites formed from the metabolism of BaP by 3-MC-, PB-, or control rat liver microsomes all have [R,R] absolute stereochemistry although the optical purity of the diol formed depends on the microsomal preparation used.

With respect to the metabolism of BaP, the presence of the peri six-methyl group does not alter the stereoselective properties of induced or non-induced rat liver MFO and EH towards the formation of trans diol metabolites, at least in terms of the absolute stereochemistry of the predominant enantiomers formed. However, the effect of the methyl group, if any, on the optical purities of the 6-MBaP trans diol metabolites formed by the various microsomal preparations could not be definitively determined by the results of this study.

Pathways of activation other than via 7,8-diol and vicinal diol epoxide intermediates have been proposed for 6-MBaP. One of these involves the metabolism of 6-MBaP to 6-OHMBaP which, in turn, is thought to be converted to an active sulfate ester (52). Although the validity of this hypothesis was not tested in the present study, 6-OHMBaP was found to be a metabolite of 6-MBaP when 3-MC-, PB-, or control microsomes were used as the source of metabolic enzymes. Other investigators have reported that 6-MBaP is metabolized to 6-OHMBaP by mouse liver microsomes and by mouse skin or rat liver homogenates (128,141), but these studies were not quantitative in nature. In the present study, 6-OHMBaP was not detected as a major metabolite of 6-MBaP with 3-MC-

microsomes presumably because it is further metabolized to 1- and 3-OH 6-OHMBaP. However, 6-OHMBaP is the major metabolite formed with PB- and control microsomes. Further metabolism of this metabolite to 6-OHMBaP phenols is minimal or does not occur when these microsomal preparations are used. Formation of the sulfate ester of 6-OHMBaP requires the presence of the liver cytosolic fraction and a sulfotransferase-PAPS generating system (52). Therefore, the relevance of the findings in the present study to this alternate proposed mechanism of 6-MBaP activation is unknown.

The possible role of the metabolites of 6-MBaP in the metabolic activation of this compound remains speculative since the biological activities of the metabolites were not tested in the present study. However, comparison of the total rates of 6-MBaP and BaP metabolite formation by 3-MC-, PB-, and control microsomes showed that rates of 6-MBaP metabolite formation are decreased by 66, 81, and 91% respectively below those of BaP metabolites. Although 6-MBaP is a weaker carcinogen than BaP, it has been found to be relatively stronger as a mutagen. BaP 4,5-oxide, precursor of the BaP 4,5-diol, is a very mutagenic compound. If the 6-MBaP 4,5-oxide is responsible for the high mutagenic activity of 6-MBaP, it would have to be extremely potent since its rate of formation in this study (as determined by 6-MBaP 4,5-diol formation) is far less than that of the BaP 4,5-diol. A possible explanation for the lower rates of 6-MBaP metabolite formation relative to those of BaP might be that, due to the presence of the six-methyl group, the orientation of the 6-MBaP substrate molecule in the active site of the mixed function oxidases (regardless of the form of cytochrome P-450 contained therein) is less favorable than the orientation achieved by the

BaP molecule (assuming both substrates remained dissolved in equal proportions under identical conditions of incubation).

Based on the results obtained in this study, portions of the pathways of 6-OHMBaP metabolism are illustrated in Fig. 32. Steps thought to involve the mixed function oxidases or epoxide hydrolase are indicated.

The original purpose for studying the microsomal metabolism of 6-OHMBaP was to determine if the 6-OHMBaP 1- and 3-phenols formed as metabolites of 6-MBaP were formed by the further metabolism of 6-OHMBaP. Indeed, the finding that 1- and 3-OH 6-OHMBaP were products of the microsomal metabolism of [^{3}H]6-OHMBaP indicated that 6-MBaP is first converted to 6-OHMBaP which is in turn metabolized to the 1- and 3-phenols of 6-OHMBaP, particularly when 3-MC-microsomes were used as the source of metabolic enzymes. Little or no 6-OHMBaP phenol formation was observed when 6-MBaP was metabolized by PB- or control microsomes. There was some conversion of [^{3}H]6-OHMBaP to 6-OHMBaP phenols by PB- and control microsomes. However, this was not surprising since the ratio of substrate to enzyme present in the incubation mixture was very high.

It cannot be concluded from the results of this study that the 6-OHMBaP 1- and 3-phenols as metabolites of 6-MBaP are formed solely as the result of the initial metabolism of 6-MBaP to 6-OHMBaP and the subsequent metabolism of 6-OHMBaP to 1-OH and 3-OH 6-OHMBaP. An alternate pathway must also be considered in which the 1- and 3-phenols of 6-MBaP are formed prior to hydroxylation of the six-methyl group of 6-MBaP. Although the possible contribution of this alternate pathway to 6-OHMBaP phenol formation cannot be determined from the results

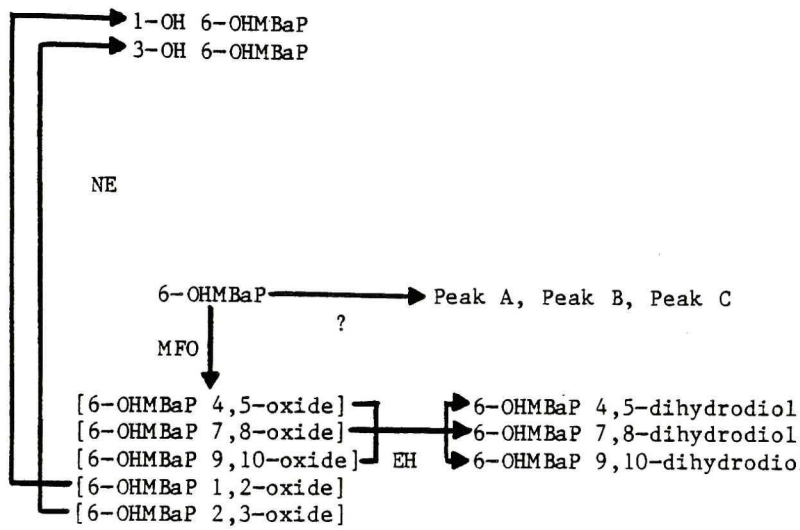


Fig. 32. In vitro metabolism of 6-OHMBaP by rat liver microsomes as elucidated in the present study. Metabolites appearing in brackets are presumed intermediates. Metabolic steps involved in the formation of Peaks A, B, and C are of course uncertain. Mixed function oxidases are abbreviated by MFO, epoxide hydrolase is abbreviated by EH, and non-enzymatic rearrangement is abbreviated by NE.

of this study, it is conceivable that once formed, both 6-OHMBaP molecules and 6-MBaP phenol molecules would have to compete with much greater numbers of 6-MBaP substrate molecules for microsomal enzymes in the incubation mixture. In experiments quantifying 6-MBaP metabolism by 3-MC-microsomes, the rate of formation of 6-MBaP phenols was found to be higher than those of the other 6-MBaP metabolites. Since they form faster, 6-MBaP phenol molecules may be relatively better competitors for further metabolism by the metabolic enzymes than are 6-OHMBaP molecules. Therefore, a pathway in which 6-MBaP is first metabolized to 1- and 3-OH 6-MBaP followed by the metabolism of either phenol to a 6-OHMBaP derivative could play a larger role in 6-OHMBaP phenol formation than the pathway (now known to be operative in vitro) in which 6-MBaP is first metabolized to 6-OHMBaP followed by the metabolism of 6-OHMBaP to the 6-OHMBaP 1- and 3-phenols.

Conversion of 6-OHMBaP to diol metabolites is a minor pathway in the in vitro metabolism of this compound by rat liver microsomes. When quantitative studies were performed, the 9,10-diol was the only diol metabolite of 6-OHMBaP to be detected. However, low rates of formation of the 6-OHMBaP 4,5- and 7,8-diols could have been obscured by a radioactive impurity (described in more detail in "Results" section). When unlabelled 6-OHMBaP, which did not contain the impurity described above, was incubated with 3-MC-microsomes for one hour, detectable amounts of 6-OHMBaP 4,5- and 7,8-diols as well as the 9,10-diol were found.

REFERENCES

1. Lee, M. L., Novotny, M. V., and Bartle, K. D. (1981). "Analytical Chemistry of Polycyclic Aromatic Compounds," pp. 17-21. Academic Press, New York.

2. Jones, P. W. (1980). Measurement and environmental impact of polycyclic aromatic hydrocarbons-some closing remarks. In "Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects" (A. Bjørseth and A. J. Dennis, eds.), pp. 1085-1090. Battelle Press, Ohio.

3. U.S. Department of Health, Education and Welfare (1967). "Smoking and Health" (Report of the Advisory Committee to the Surgeon General of the Public Health Service), Public Health Serv. Publ. No. 1103. US Govt. Printing Office, Washington, D.C.

4. Lijinsky, W., and Shubik, P. (1964). Benzo[a]pyrene and other polynuclear hydrocarbons in charcoal-broiled meat. Science 145, 53-55.

5. Santodonato, J., Basu, D., and Howard, P. H. (1980). Multimedia human exposure and carcinogenic risk assessment for environmental PAH. In "Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects" (A. Bjørseth and A. J. Dennis, eds.), pp. 435-454. Battelle Press, Ohio.

6. Kaiser, C., Kerr, A., McCalla, D. R., Lockington, J. N., and Gibson, E. S. (1980). Mutagenic material in air particles in a steel foundry. In "Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects" (A. Bjørseth and A. J. Dennis, eds.), pp. 579-588. Battelle Press, Ohio.

7. Dipple, A. (1976). Polynuclear aromatic carcinogens. In "Chemical Carcinogens" (C. E. Searle, ed.), pp. 245-314. American Chemical Society Monograph No. 173, Washington, D.C.

8. Sims, P. and Grover, P. L. (1974). Epoxides in polycyclic aromatic hydrocarbon metabolism and carcinogenesis. Adv. Cancer Res. 20, 165-274.

9. Vainio, H., and Hietanen, E. (1980). Role of extrahepatic metabolism. In "Concepts in Drug Metabolism, Part A" (P. Jenner and B. Testa, eds.), pp. 251-284. Marcel Dekker, New York.

10. Alvares, A. P. (1982). Oxidative biotransformation of drugs. In "The Liver: Biology and Pathobiology" (I. M. Arias, H. Popper, D. Schachter, and D. A. Shafritz, eds.), pp. 265-280. Raven Press, New York.

11. Estabrook, R. W., Werringloer, J., Capedevila, J., and Prough, R. A. (1978). The role of cytochrome P-450 and the microsomal electron transport system: the oxidative metabolism of benzo-

[a]pyrene. In "Polycyclic Hydrocarbons and Cancer" (H. V. Gelboin and P. O. P. Ts'o, eds.), Vol. 1, pp. 285-319. Academic Press, New York.

12. Kappas, A., and Alvares, A. P. (1975). How the liver metabolizes foreign substances. Scientific American 232, 22-31.

13. Estabrook, R. W., and Werringloer, J. (1977). Cytochrome P-450 - its role in oxygen activation for drug metabolism. In "Drug Metabolism Concepts" (D. M. Jerina, ed.), American Chemical Society Symposium Series 44, pp. 1-25. American Chemical Society, Washington, D.C.

14. Strobel, H. W., Dignam, J. D., and Gum, J. R. (1980). NADPH cytochrome P-450 reductase and its role in the mixed function oxidase reaction. Pharmac. Ther. 8, 525-537.

15. Yang, S. K., Deutsch, J., and Gelboin, H. V. (1978). Benzo[a]-pyrene metabolism: activation and detoxification. In "Polycyclic Hydrocarbons and Cancer" (H. V. Gelboin and P. O. P. Ts'o, eds.), Vol. 1, pp. 205-231. Academic Press, New York.

16. Yang, S. K., Roller, P. P., Fu, P.P., Harvey, R. G., and Gelboin, H. V. (1977). Evidence for a 2,3-epoxide as an intermediate in the microsomal metabolism of benzo[a]pyrene to 3-hydroxy-benzo[a]pyrene. Biochem. Biophys. Res. Commun. 77, 1176-1182.

17. Holder, G., Yagi, H., Dansette, P., Jerina, D. M., Levin, W., Lu, A. Y. H., and Conney, A. H. (1974). Effects of inducers and epoxide hydrase on the metabolism of benzo[a]pyrene by liver microsomes and a reconstituted system: analysis by high pressure liquid chromatography. Proc. Nat. Acad. Sci. USA 71, 4356-4360 and references therein.

18. Jerina, D. M., and Daly, J. W. (1974). Arene oxides: a new aspect of drug metabolism. Science 185, 573-581 and references therein.

19. Jerina, D. M. and Daly, J. W. (1976). Oxidation at carbon. In "Drug Metabolism - From Microbe to Man" (D. W. Parke and R. L. Smith, eds.), pp. 13-32. Taylor and Francis, London.

20. Daly, J. W., Jerina, D. M., and Witkop, B. (1972). Arene oxides and the NIH shift: the metabolism, toxicity and carcinogenicity of aromatic compounds. Experientia 28, 1129-1149.

21. Lu, A. Y. H., and Miwa, G. T. (1980). Molecular properties and biological functions of microsomal epoxide hydrase. Ann. Rev. Pharmacol. Toxicol. 20, 513-531.

22. Yang, S. K., Roller, P. P., and Gelboin, H. V. (1977). Enzymatic mechanism of benzo[a]pyrene conversion to phenols and diols and an improved high-pressure liquid chromatographic separation of benzo[a]pyrene derivatives. Biochemistry 16, 3680-3687.

23. Selkirk, J. K., Croy, R. G., Roller, P. P., and Gelboin, H. V. (1974). High-pressure liquid chromatographic analysis of benzo[a]pyrene metabolism and covalent binding and the mechanism of action of 7,8-benzoflavone and 1,2-epoxy-3,3,3-trichloropropane. Can. Res. 34, 3474-3480.

24. Croy, R. G., Selkirk, J. K., Harvey, R. G., Engel, J. F., and Gelboin, H. V. (1976). Separation of ten benzo[a]pyrene phenols by recycle high pressure liquid chromatography and identification of four phenols as metabolites. Biochem. Pharmacol. 25, 227-230.

25. Selkirk, J. K., Croy, R. G., and Gelboin, H. V. (1976). High pressure liquid chromatographic separation of ten benzo[a]-pyrene phenols and the identification of 1-phenol and 7-phenol as new metabolites. Can. Res. 36, 922-926.

26. Yang, S. K., Selkirk, J. K., Plotkin, E. V., and Gelboin, H. V. (1975). Kinetic analysis of the metabolism of benzo[a]pyrene to phenols, dihydrodiols, and quinones by high-pressure chromatography compared to analysis by aryl hydrocarbon hydroxylase assay, and the effect of enzyme induction. Can. Res. 35, 3642-3650.

27. Falk, H. L., Kotin, P., Lee, S. S., and Nathan, A. (1962). Intermediary metabolism of benzo[a]pyrene in the rat. J. Natl. Cancer Inst. 28, 699-724.

28. Levin, W., Wood, A., Chang, R., Ryan, D., Thomas, P., Yagi, H., Thakker, D., Vyas, K., Boyd, C., Chu, S.-Y., Conney, A., and Jerina, D. (1982). Oxidative metabolism of polycyclic aromatic hydrocarbons to ultimate carcinogens. Drug Metabolism Reviews 13, 555-580.

29. Nagata, C., Kodama, M., and Ioki, Y. (1978). Electron spin resonance study of the binding of the 6-oxybenzo[a]pyrene radical and benzo[a]pyrene semiquinone radicals with DNA and polynucleotides. In "Polycyclic Hydrocarbons and Cancer" (H. B. Gelboin and P. O. P. Ts'o, eds.), Vol. 1, pp. 247-259. Academic Press, New York.

30. Lesko, S., Caspary, W., Lorentzen, R., and Ts'o, P. O. P. (1975). Enzymic formation of 6-oxobenzo[a]pyrene radical in rat liver homogenates from carcinogenic benzo[a]pyrene. Biochemistry 14, 3978-3983.

31. Nagata, C., Inomata, M., Kodama, M., and Tagashira, Y. (1968). Electron spin resonance study on the interaction between the chemical carcinogens and tissue components. III. Determination of the structure of the free radical produced either by stirring 3,4-benzopyrene with albumin or incubating it with liver homogenate. Gann 59, 289-298.

32. Lorentzen, R. J., Caspary, W. J., Lesko, S. A., and Ts'o, P. O. P. (1975). The autooxidation of 6-hydroxybenzo[a]pyrene and 6-oxobenzo[a]pyrene radical, reactive metabolites of benzo[a]pyrene. Biochemistry 14, 3970-3977.

33. Selkirk, J. K., Croy, R. G., and Gelboin, H. V. (1974). Benzo[a]-pyrene metabolites: efficient and rapid separation by high-pressure liquid chromatography. Science 184, 169-171.

34. Cavalieri, E. L., and Rogan, E. G. (in press). One-electron and two-electron oxidation in aromatic hydrocarbon carcinogenesis. In "Free Radicals in Biology" (W. A. Pryor, ed.), Vol. VI. Academic Press, New York.

35. Sims, P. (1967). The metabolism of benzo[a]pyrene by rat-liver homogenates. Biochem. Pharmacol. 16, 613-618.

36. Wiebel, F. J. (1975). Metabolism of monohydroxybenzo[a]pyrenes by rat liver microsomes and mammalian cells in culture. Arch. Biochem. Biophys. 168, 609-621.

37. Yang, S. K., McCourt, D. W., Roller, P. P., and Gelboin, H. V. (1976). Enzymatic conversion of benzo[a]pyrene leading predominantly to the diol-epoxide r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene through a single enantiomer of r-7,t-8-dihydroxy-7,8-dihydrobenzo[a]pyrene. Proc. Natl. Acad. Sci. USA 73, 2594-2598.

38. Thakker, D. R., Yagi, H., Lu, A. Y. H., Levin, W., Conney, A. H., and Jerina, D. M. (1976). Metabolism of benzo[a]pyrene: conversion of (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene to highly mutagenic 7,8-diol-9,10-epoxides. Proc. Natl. Acad. USA 73, 3381-3385.

39. Thakker, D. R., Yagi, H., Akagi, H., Koreeda, M., Lu, A. Y. H., Levin, W., Wood, A. W., Conney, A. H., and Jerina, D. M. (1977). Metabolism of benzo[a]pyrene. VI. Stereoselective metabolism of benzo[a]pyrene and benzo[a]pyrene 7,8-dihydrodiol to diol epoxides. Chem.-Biol. Interactions 16, 281-300.

40. Caldwell, J. (1982). Conjugation reactions in the metabolism of xenobiotics. In "The Liver: Biology and Pathobiology" (I. Arias, H. Popper, D. Schachter, and D. A. Shafritz, eds.), pp. 281-295. Raven Press, New York.

41. Nemoto, N. and Takayama, S. and Gelboin, H. V. (1977). Enzymatic conversion of benzo[a]pyrene phenols, dihydrodiols and quinones to sulfate conjugates. Biochem. Pharmacol. 26, 1825-1829.

42. Nemoto, N. and Gelboin, H. V. (1976). Enzymatic conjugation of benzo[a]pyrene oxides, phenols, and dihydrodiols with UDP-glucuronic acid. Biochem. Pharmacol. 25, 1221-1226.

43. Jakoby, W. B. (1978). The glutathione S-transferases: a group of multifunctional detoxification proteins. In "Advances in Enzymology and Related Areas of Molecular Biology" (A. Meister, ed.), pp. 383-414. John Wiley and Sons, New York.

44. Sims, P. (1980). Dihydrodiols and diol-epoxides in the activation and detoxification of polycyclic hydrocarbons. In "Carcinogenesis: Fundamental Mechanisms and Environmental Effects" (B. Pullman, P. O. P. Ts'o, and H. V. Gelboin, eds.), pp. 33-42. D. Reidel, New York.

45. Schelin, C., Tunek, A., Jernström, B., and Jergil, B. (1980). Irreversible binding of isolated benzo[a]pyrene metabolites to specific rat liver microsomal proteins. Mol. Pharmacol. 18, 529-535.

46. Brookes, P., Newbold, R. F., and Osborne, M. R. (1979). Mechanism of the carcinogenicity of polycyclic hydrocarbons. In "Environmental Carcinogenesis" (P. Emmelot and E. Kriek, eds.), pp. 123-142. Elsevier/North-Holland Biomedical Press, Amsterdam.

47. Yang, S. K., Gelboin, H. V., Trump, B. F., Autrup, H., and Harris, C. C. (1977). Metabolic activation of benzo[a]pyrene and binding to DNA in cultured human bronchus. Can. Res. 37, 1210-1215.

48. Gelboin, H. V., Kinoshita, N., and Wiebel, F. J. (1972). Microsomal hydroxylases: induction and role in polycyclic hydrocarbon carcinogenesis and toxicity. Fed. Proceedings 31, 1298-1309.

49. Miller, J. A., and Miller, E. C. (1976). The metabolic activation of chemical carcinogens to reactive electrophiles. In "Biology of Radiation Carcinogenesis" (J. M. Yuhas, R. W. Tennant, and J. D. Regan, eds.), pp. 147-164. Raven Press, New York.

50. Caldwell, J. (1979). The significance of phase II (conjugation) reactions in drug disposition and toxicity. Life Sciences 24, 571-578.

51. Miller, E. C., (1978). Some current perspectives on chemical carcinogenesis in humans and experimental animals: presidential address. Can. Res. 38, 1479-1496 and references therein.

52. Flesher, J. W., Kadry, A. M., Chien, M., Stansbury, K. H., Gairola, C., and Sydnor, K. L. (1983). Metabolic activation of carcinogenic hydrocarbons in the meso position (L-region). In "Polynuclear Aromatic Hydrocarbons: Formation, Metabolism and Measurement" (M. Cooke and A. J. Dennis, eds.), Seventh International Symposium, pp. 505-515. Battelle Press, Ohio.

53. Conney, A. H. (1980). Microsomes and drug oxidations: perspectives and challenges. In "Microsomes, Drug Oxidation, and Chemical Carcinogenesis, Vol. II" (M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gillette, and P. J. O'Brien, eds.), pp. 1103-1118. Academic Press, New York.

54. Conney, A. H. (1982). Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. Can. Res. 42, 4875-4917.

55. Pelkonen, O. and Nebert, D. W. (1982). Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. Pharmacol. Reviews 34, 189-222.

56. Alvares, A. P., Schilling, G., Levin, W., and Kuntzman, R. (1967). Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methylcholanthrene. Biochem. Biophys. Res. Commun. 29, 521-526.

57. Sladek, N. E. and Mannering, G. J. (1966). Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Commun. 24, 668-674.

58. Rogan, E. G., Katomski, P. A., Roth, R. W., and Cavalieri, E. L. (1979). Horseradish peroxidase/hydrogen peroxide-catalyzed binding of aromatic hydrocarbons to DNA. J. Biolog. Chem. 254, 7055-7059.

59. Thakker, D. R., Levin, W., Yagi, H., Conney, A. H., and Jerina, D. M. (1982). Regio- and stereoselectivity of hepatic cytochrome P-450 toward polycyclic aromatic hydrocarbon substrates. In "Advances in Experimental Medicine and Biology" (R. Snyder, D. V. Parke, J. V. Kocsis, D. J. Jollow, C. G. Gibson, and C. M. Wither, eds.), Vol. 136A, pp. 525-539. Plenum Press, New York.

60. Guengerich, F. P. (1979). Isolation and purification of cytochrome P-450, and the existence of multiple forms. Pharmac. Ther. 6, 99-121.

61. Lu, A. Y. H., and West, S. B. (1980). Multiplicity of mammalian microsomal cytochromes P-450. Pharmacol. Reviews 31, 277-295.

62. Ryan, D. E., Thomas, P. E., Reik, L. M., and Levin, W. (1982). Purification, characterization and regulation of five rat hepatic microsomal cytochrome P-450 isozymes. Xenobiotica 12, 727-744.

63. Ryan, D. E., Thomas, P. E., Korzeniowski, D., and Levin, W. (1979). Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. J. Biolog. Chem. 254, 1365-1374.

64. Alvares, A. P., Schilling, G., Levin, W., and Kuntzman, R. (1968). Alteration of the microsomal hemoprotein by 3-methylcholanthrene: effects of ethionine and actinomycin D. J. Pharmacol. Exp. Ther. 163, 417-424.

65. Bhat, K. S. and Padmanaban, G. (1979). Studies on the biosynthesis of cytochrome P-450 in rat liver - a probe with phenobarbital. Arch. Biochem. Biophys. 198, 110-116.

66. Dubois, R. N., and Waterman, M. R. (1979). Effect of phenobarbital administration to rats on the level of the in vitro synthesis of cytochrome P-450 directed by total rat liver RNA. Biochem. Biophys. Res. Commun. 90, 150-157.

67. Poland, A., Glover, E., and Kende, A. S. (1976). Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. J. Biol. Chem. 251, 4936-4946.

68. Fouts, J. R., and Rogers, L. A. (1965). Morphological changes in the liver accompanying stimulation of microsomal drug metabolizing enzyme activity by phenobarbital, chlordane, benzpyrene or methylcholanthrene in rats. J. Pharmacol. Exp. Ther. 147, 112-119.

69. Lu, A. Y. H., Levin, W., Vore, M., Conney, A. H., Thakker, D. R., Holder, G., and Jerina, D. M. (1976). Metabolism of benzo[a]pyrene by purified liver microsomal cytochrome P-448 and epoxide hydrase. In "Carcinogenesis, Vol. 1, Polynuclear Aromatic Hydrocarbons: Chemistry, Metabolism and Carcinogenesis" (R. I. Freudenthal and P. W. Jones, eds.), pp. 115-126, Raven Press, New York.

70. Wiebel, F. J., Selkirk, J. K., Gelboin, H. V., Haugen, D. A., van der Hoeven, T. A., and Coon, M. J. (1975). Position-specific oxygenation of benzo[a]pyrene by different forms of purified cytochrome P-450 from rabbit liver. Proc. Nat. Acad. Sci. USA 72, 3917-3920.

71. Deutsch, J., Leutz, J. C., Yang, S. K., Gelboin, H. V., Chiang, Y. L., Vatsis, K. P., and Coon, M. J. (1978). Regio- and stereoselectivity of various forms of purified cytochrome P-450 in the metabolism of benzo[a]pyrene and (-)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene as shown by product formation and binding to DNA. Proc. Natl. Acad. Sci. USA 75, 3123-3127.

72. Leutz, J. C., and Gelboin, H. V. (1975). Benzo[a]pyrene-4,5-oxide hydratase: assay, properties, and induction. Arch. Biochem. Biophys. 168, 722-725.

73. Bresnick, E., Muktar, H., Stoming, T. A., Dansette, P. M., and Jerina, D. M. (1977). Effect of phenobarbital and 3-methylcholanthrene administration on epoxide hydrase levels in liver microsomes. Biochem. Pharmacol. 26, 891-892.

74. Kasper, C. B. and Henton, D. (1980). Glucuronidation. In "Enzymatic Basis of Detoxication, Vol. II" (W. B. Jakoby, ed.), pp. 3-36. Academic Press, New York.

75. Yang, S. K., McCourt, D. W., Leutz, J. C., Gelboin, H. V. (1977). Benzo[a]pyrene diol epoxides: mechanism of enzymatic formation and optically active intermediates. Science 196, 1199-1201.

76. Thakker, D. R., Yagi, H., Levin, W., Lu, A. Y. H., Conney, A. H., and Jerina, D. M. (1977). Stereospecificity of microsomal and purified epoxide hydrase from rat liver. Hydration of arene oxides of polycyclic hydrocarbons. J. Biolog. Chem. 252, 6328-6334.

77. Nordquist, M., Thakker, D. R., Levin, W., Yagi, H., Ryan, D. E., Thomas, P. E., Conney, A. H., and Jerina, D. M. (1979). The highly tumorigenic 3,4-dihydrodiol is a principal metabolite formed from dibenzo[a,h]anthracene by liver enzymes. Mol. Pharmacol. 16, 643-655.

78. Jerina, D. M., Yagi, H., Thakker, D. R., Karle, J. M., Mah, H. D., Boyd, D. R., Gadaginamath, G., Wood, A. W., Buening, M., Chang, R. L., Levin, W., and Conney, A. H. (1979). Stereoselective metabolic activation of polycyclic aromatic hydrocarbons. In "Advances in Pharmacology and Therapeutics" (Y. Cohen, ed.), Vol. 9, pp. 53-61. Pergamon Press, Oxford.

79. Buening, M. K., Wislocki, P. G., Levin, W., Yagi, H., Thakker, D. R., Akagi, H., Koreeda, M., Jerina, D. M., and Conney, A. H. (1978). Tumorigenicity of the optical enantiomers of the diastereomeric benzo[a]pyrene 7,8-diol-9,10-epoxides in newborn mice: exceptional activity of (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. Proc. Natl. Acad. Sci. USA 75, 5358-5361.

80. Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Thakker, D. R., Jerina, D. M., and Conney, A. H. (1977). Differences in mutagenicity of the optical enantiomers of the diastereomeric benzo[a]pyrene 7,8-diol-9,10-epoxides. Biochem. Biophys. Res. Commun. 77, 1389-1396.

81. Jerina, D. M., Sayer, J. M., Thakker, D. R., Yagi, H., Levin, W., Wood, A. W., and Conney, A. H. (1980). Carcinogenicity of polycyclic aromatic hydrocarbons: the bay region theory. In "Carcinogenesis: Fundamental Mechanisms and Environmental Effects" (B. Pullman, P. O. P. Ts'o, and H. Gelboin, eds.), pp. 1-12. D. Reidel Publishers, Boston, MA.

82. Kinoshita, N., Shears, B., and Gelboin, H. V. (1973). K-region and non-K-region metabolism of benzo[a]pyrene by rat liver microsomes. Can. Res. 33, 1937-1944.

83. Levin, W., Wood, A. W. Lu, A. Y. H., Ryan, D., West, S., Conney, A. H., Thakker, D. R., Yagi, H., Jerina, D. M. (1977). Role of purified cytochrome P-448 and epoxide hydrase in the activation and detoxification of benzo[a]pyrene. In "Drug Metabolism Concepts" (D. M. Jerina, ed.), A. C. S. Symposium Series 44, pp. 99-125. American Chemical Society, Washington, D.C.

84. Jerina, D. M., Michaud, D. P., Feldmann, R. J., Armstrong, R. N., Vyas, K. P., Thakker, D. R., Yagi, H., Thomas, P. E., Ryan, D. E., Levin, W. (1982). Stereochemical modeling of the catalytic site of cytochrome P-450$_c$. In "Microsomes, Drug Oxidations and Drug Toxicology" (R. Sato and R. Kato, eds.), pp. 195-201. Wiley - Interscience, New York.

85. Yang, S. K., Roller, P.P., and Gelboin, H. V. (1978). Benzo[a]-pyrene metabolism: mechanism in the formation of epoxides, phenols, dihydrodiols and the 7,8-diol-9,10-epoxides. In "Carcinogenesis, Vol. 3: Polynuclear Aromatic Hydrocarbons" (P. W. Jones and R. I. Freudenthal, eds.), pp. 285-301. Raven Press, New York.

86. Jerina, D. M., Lehr, R., Schaefer-Ridder, M., Yagi, H., Karle, J. M., Thakker, D. R., Wood, A. W., Lu, A. Y. H., Ryan, D., West, S., Levin, W., and Conney, A. H. (1977). Bay-region epoxides of dihydrodiols: a concept explaining the mutagenic and carcinogenic activity of benzo[a]pyrene and benzo[a]anthracene. In "Origins of Human Cancer" (H. H. Hiatt and J. D. Watson, eds.), pp. 639-658, Cold Spring Harbor Laboratory, New York.

87. Levin, W., Wood, A. W., Wislocki, P. G., Chang, R. L., Kapitulnik, J., Mah, H. D., Yagi, H., Jerina, D. M., and Conney, A. H. (1978). Mutagenicity and carcinogenicity of benzo[a]pyrene and benzo[a]pyrene derivatives. In "Polycyclic Hydrocarbons and Cancer" (H. V. Gelboin and P. O. P. Ts'o, eds.), Vol. 1, pp. 189-202. Academic Press, New York

88. Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Hernandez, O., Dansette, P. M., Jerina, D. M., and Conney, A. H. (1976). Mutagenicity and cytotoxicity of benzo[a]pyrene arene oxides, phenols, quinones, and dihydrodiols in bacterial and mammalian cells. Can. Res. 36, 3350-3357.

89. Wood, A. W., Levin, W., Lu, A. Y. H., Yagi, H., Hernandez. O., Jerina, D. M., and Conney, A. H. (1976). Metabolism of benzo-[a]pyrene and benzo[a]pyrene derivatives to mutagenic products by highly purified hepatic microsomal enzymes. J. Biol. Chem. 251, 4882-4890.

90. Huberman, E., Sachs, L., Yang, S. K., and Gelboin, H. V. (1976). Identification of mutagenic metabolites of benzo[a]pyrene in mammalian cells. Proc. Nat. Acad. Sci. USA 73, 607-611.

91. Conney, A. H., Levin, W., Wood, A. W., Yagi, H., Thakker, D. R., Lehr, R. E., and Jerina, D. M. (1980). Metabolism of polycyclic aromatic hydrocarbons to reactive intermediates with high biological activity. In "Human Epidemiology and Animal Laboratory Correlations in Chemical Carcinogenesis" (F. Coulston and P. Shubik, eds.), pp. 153-183. Ablex Publishing Corporation, Norwood, New Jersey.

92. Wood, A. W., Wislocki, P. G., Chang, R. L., Levin, W., Lu, A. Y. H., Yagi, H., Hernandez, O., Jerina, D. M., and Conney, A. H. (1976). Mutagenicity and cytotoxicity of benzo[a]pyrene benzo-ring epoxides. Can. Res. 36, 3358-3366.

93. Levin, W., Wood, A. W. Wislocki, P. G. Kapitulnik, J., Yagi, H., Jerina, D. M., and Conney, A. H. (1977). Carcinogenicity of benzo-ring derivatives of benzo[a]pyrene on mouse skin. Can. Res. 37, 3356-3361.

94. Levin, W., Wood, A. W., Yagi, H., Jerina, D. M., and Conney, A. H. (1976). (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene: a potent skin carcinogen when applied topically to mice. Proc. Natl. Acad. Sci. USA 73, 3867-3871.

95. Kapitulnik, J., Levin, W., Conney, A. H., Yagi, H., Jerina, D. M. (1977). Benzo[a]pyrene 7,8-dihydrodiol is more carcinogenic than benzo[a]pyrene in newborn mice. Nature (Lond.) 266, 378-380.

96. Slaga, T. J., Bracken, W. M., Viaje, A., Berry, D. L., Fischer, S. M., Miller, D. R., Levin, W., Conney, A. H., Yagi, H., and Jerina, D. M. (1978). Tumor initiating and promoting activities of various benzo[a]pyrene metabolites in mouse skin. In "Carcinogenesis, Vol. 3: Polynuclear Aromatic Hydrocarbons" (P. W. Jones and R. I. Freudenthal, eds.), pp. 371-382. Raven Press, New York.

97. Kapitulnik, J., Wislocki, P. G., Levin, W., Yagi, H., Jerina, D. M., and Conney, A. H. (1978). Tumorigenicity studies with diol-epoxides of benzo[a]pyrene which indicate that (±)-trans-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene' is an ultimate carcinogen in newborn mice. Can. Res. 38., 354-358.

98. Levin, W., Wood, A. W., Yagi, H., Dansette, P. M., Jerina, D. M., and Conney, A. H. (1976). Carcinogenicity of benzo[a]pyrene 4,5-, 7,8-, and 9,10-oxides on mouse skin. Proc. Nat. Acad. Sci. USA 73, 243-247.

99. Kapitulnik, J., Levin, W., Yagi, H., Jerina, D.M., and Conney, A. H. (1976). Lack of carcinogenicity of 4-, 5-, 6-, 7-, 8-, 9-, and 10-hydroxybenzo[a]pyrene on mouse skin. Can. Res. 36, 3625-3628.

100. Wislocki, P. G., Chang, R. L., Wood, A. W., Levin, W., Yagi, H., Hernandez, O., Mah, H. D., Dansette, P. M., Jerina, D. M., and Conney, A. H. (1977). High carcinogenicity of 2-hydroxybenzo-[a]pyrene on mouse skin. Can. Res. 37, 2608-2611.

101. Levin, W., Buening, M. K., Wood, A. W., Chang, R. L., Kedzierski, B., Thakker, D. R., Boyd, D. R., Gadaginamath, G. S., Armstrong, R. N., Yagi, H., Karle, J. M., Slaga, T. J., Jerina, D. M., and Conney, A. H. (1980). An enantiomeric interaction in the metabolism and tumorigenicity of (+)- and (-)-benzo[a]-pyrene 7,8-oxide. J. Biol. Chem. 255, 9067-9074.

102. Kapitulnik, J., Wislocki, P. G., Levin, W., Yagi, H., Thakker, D. R., Akagi, H., Koreeda, M., Jerina, D. M., and Conney, A. H. (1978). Marked differences in the carcinogenic activity of optically pure (+)- and (-)-trans-7,8-dihydroxy-7,8-dihydro-benzo[a]pyrene in newborn mice. Can. Res. 38, 2661-2665.

103. Levin, W., Wood, A. W., Chang, R. L., Slaga, T. J., Yagi, H., Jerina, D. M., and Conney, A. H. (1977). Marked differences in the tumor-initiating activity of optically pure (+)- and (-)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene on mouse skin. Can. Res. 37, 2721-2725.

104. Slaga, T. J., Bracken, W. J., Gleason, G., Levin, W., Yagi, H., Jerina, D. M., and Conney, A. H. (1979). Marked differences in the skin tumor-initiating activities of the optical enantiomers of the diastereomeric benzo[a]pyrene 7,8-diol-9,10-epoxides. Can. Res. 39, 67-71.

105. Koreeda, M., Moore, P. D., Wislocki, P. G., Levin, W., Conney, A. H., Yagi, H., and Jerina, D. M. (1978). Binding of benzo-[a]pyrene 7,8-diol-9,10-epoxides to DNA, RNA, and protein of mouse skin occurs with high stereoselectivity. Science 199, 778-781.

106. Moore, P. D., Koreeda, M., Wislocki, P. G., Levin, W., Conney, A. H., Yagi, H., and Jerina, D. M. (1977). In vitro reactions of the diastereomeric 9,10-epoxides of (+)- and (-)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene with polyguanylic acid and evidence for formation of an enantiomer of each diastereomeric 9,10-epoxide from benzo[a]pyrene in mouse skin. In "Drug Metabolism and Concepts" (D. M. Jerina, ed.), American Chemical Society Symposium Series, No. 44, pp. 127-154. American Chemical Society, Washington, D.C.

107. Meehan, T., and Straub, K. (1979). Double-stranded DNA stereo-selectively binds benzo[a]pyrene diol epoxides. Nature 277, 410-412.

108. Jerina, D. M., Yagi, H., Lehr, R. E., Thakker, D. R., Schaefer-Ridder, M., Karle, J. M., Levin, W., Wood, A. W., Chang, R. L., and Conney, A. H. (1978). The bay-region theory of carcino-genesis by polycyclic aromatic hydrocarbons. In "Polycyclic

Hydrocarbons and Cancer" (H. V. Gelboin and P. O. P. Ts'o, eds.), Vol. 1, pp. 173-187. Academic Press, New York.

109. Borgen, A., Darvey, H., Castagnoli, N., Crocker, T. T., Rasmussen, R. E., and Wang, I. Y. (1973). Metabolic conversion of benzo-[a]pyrene by Syrian hamster liver microsomes and binding of metabolites to deoxyribonucleic acid. J. Med. Chem. 16, 502-506.

110. Sims, P., Grover, P. L., Swaisland, A., Pal, K., and Hewer, A. (1974). Metabolic activation of benzo[a]pyrene proceeds by a diol-epoxide. Nature 252, 326-328.

111. Jerina, D. M., and Lehr, R. E. (1977). The bay-region theory: a quantum mechanical approach to aromatic hydrocarbon-induced carcinogenicity. In "Microsomes and Drug Oxidations" (V. Ullrich, A. Hildebrandt, I. Roots, R. W. Estabrook, and A. H. Conney, eds.), pp. 709-720. Pergamon Press, New York.

112. Dewer, M. J. S. (1969). "The Molecular Orbital Theory of Organic Chemistry," pp. 214 and 304. McGraw-Hill, New York.

113. R. E. Lehr, Levin, W., Wood, A. W., Conney, A. H., Yagi, H., and Jerina, D. M. (1977). Quantum chemically based predictions of polycyclic aromatic hydrocarbon carcinogenicity - the bay region theory. In "Structural Correlates of Carcinogenesis and Mutagenesis" (I. M. Asher and C. Zervos, eds.), Proceedings of the Second FDA Office of Science Summer Symposium, pp. 129-135. The Office of Science, FDA, Rockville, Md.

114. Wood, A. W., Levin, W., Thakker, D. R., Yagi, H., Chang, R. L., Ryan, D. E., Thomas, P. E., Dansette, P. M., Whittaker, N., Turujman, S., Lehr, R. E., Kumar, S., Jerina, D. M., and Conney, A. H. (1979). Biological activity of benzo[e]pyrene J. Biol. Chem. 254, 4408-4415.

115. Rogan, E., Roth, R., and Cavalieri, E. (1979). Binding of aromatic hydrocarbons to DNA catalyzed by peroxidase and by ATP. In "Polynuclear Aromatic Hydrocarbons" (P. W. Jones and P. Leber, eds.), pp. 685-694. Ann Arbor Science Publishers, Ann Arbor, MI., and references therein.

116. Cavalieri, E., Roth, R., and Rogan, E. (1979). Hydroxylation and conjugation at the benzylic carbon atom: a possible mechanism of activation for some methyl substituted aromatic hydrocarbons. In "Polynuclear Aromatic Hydrocarbons" (P. W. Jones and P. Leber, eds.), pp. 517-529. Ann Arbor Science Publishers, Ann Arbor, MI., and references therein.

117. Cavalieri, E., Roth, R., Rogan, E., Grandjean, C., and Althoff, J. (1978). Mechanisms of tumor initiation by polycyclic aromatic hydrocarbons. In "Carcinogenesis, Vol. 3: Polynuclear Aromatic Hydrocarbons" (P. W. Jones and R. I. Freudenthal, eds.), pp. 273-284, Raven Press, New York.

118. Fieser, L. F. and Hershberg, E. B. (1939). The orientation of 3,4-benzpyrene in substitution reactions. J. Am. Chem. Soc. 61, 1565-1574.

119. Jeftic, L., and Adams, R. N. (1970). Electrochemical oxidation pathways of benzo[a]pyrene. J. Am. Chem. Soc. 92, 1332-1337.

120. Caspary, W., Cohen, B., Lesko, S., and Ts'o, P. O. P. (1973). Electron paramagnetic resonance study of iodine-induced radicals of benzo[a]pyrene and other polycyclic hydrocarbons. Biochemistry 12, 2649-2656.

121. Menger, E. M., Spokane, R. B., and Sullivan, P. D. (1976). Free radicals derived from benzo[a]pyrene. Biochem. Biophys. Res. Commun. 71, 610-616.

122. Rogan, E., Roth, R., Katomski, P., Benderson, J., and Cavalieri, E. (1978). Binding of benzo[a]pyrene at the 1, 3, 6 positions to nucleic acids in vivo on mouse skin and in vitro with rat liver microsomes and nuclei. Chem.-Biol. Interactions 22, 35-51.

123. Kodama, M., and Nagata, C. (1977). Binding of 6-oxybenz[a]pyrene radical with DNA and polynucleotides. Gann 68, 125-126.

124. Lacassagne, A., Zajdela, F., Buu-Hoï, N. P., Chalvet, O., and Daub, G. H. (1968). High carcinogenic activity of mono-, di-, and trimethylbenzo[a]pyrenes. Int. J. Cancer 3, 238-243.

125. Schürch, O., and Winsterstein, A. (1935). Über die krebserregende Wirkung Aromatischer Kohlenwasserstoffe. Ztschr. Physiol. Chem., 79-91.

126. Arcos, J. C. and Argus, M. F. (1974). "Chemical Induction of Cancer." Section 5.1.1.2. Academic Press, New York.

127. Iyer, R. P., Lyga, J. W., Secrist, III., J. A., Daub, G. H., and Slaga, T. J. (1980). Comparative tumor-initiating activity of methylated benzo[a]pyrene derivatives in mouse skin. Can. Res. 40, 1073-1076.

128. Cavalieri, E., Roth, R., Grandjean, C., Althoff, J., Patil, K., Liakus, S., and Marsh, S. (1978). Carcinogenicity and metabolic profiles of 6-substituted benzo[a]pyrene derivatives on mouse skin. Chem.-Biol. Interactions 22, 53-67.

129. Sydnor, K. L., Bergo, C. H., and Flesher, J. W. (1980). Effect of various substituents in the 6-position on the relative carcinogenic activity of a series of benzo[a]pyrene derivatives. Chem.-Biol. Interactions 29, 159-167.

130. Chiu, P.-L., and Yang, S. K. (1982). A structure-activity relationship study of monomethylbenzo[a]pyrenes by the use of

Salmonella typhimurium tester strain TA100 and by analysis of metabolite formation. In "Polynuclear Aromatic Hydrocarbons: Physical and Biological Chemistry" (M. Cooke, A. S. Dennis, and G. L. Fisher, eds.), pp. 193-200. Battelle Press, Ohio.

131. Santella, R., Kinoshita, T., and Jeffrey, A. M. (1982). Mutagenicity of some methylated benzo[a]pyrene derivatives. Mut. Res. 104, 209-213.

132. Hecht, S. S., Mazzarese, R., Amin, S., LaVoie, E., and Hoffman, D. (1979). On the metabolic activation of 5-methylchrysene. In "Polycyclic Aromatic Hydrocarbons" (P. W. Jones and P. Leber, eds.), pp. 733-752. Ann Arbor Science Publishers, Ann Arbor, MI.

133. Tierney, B., Burden, P. Hewer, A., Ribeiro, O., Walsh, C., Rattle, H., Grover, P. L., and Sims, P. (1979). High-performance liquid chromatography of isomeric dihydrodiols of polycyclic hydrocarbons. J. Chromatography 176, 329-335.

134. Thakker, D. R., Yagi, H., Lehr, R. E., Levin, W., Buening, M., Lu, A. Y. H., Chang, R. L., Wod, A. W., Conney, A. H., and Jerina, D. M. (1978). Metabolism of trans-9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene occurs primarily by aryl hydroxylation rather than formation of a diol epoxide. Mol. Pharmacol. 14, 502-513.

135. Yang, S. K., Chou, M. W., and Fu, P. P. (1980). Metabolic and structural requirements for the carcinogenic potencies of unsubstituted and methyl-substituted polycyclic aromatic hydrocarbons. In "Carcinogenesis: Fundamental Mechanisms and Environmental Effects" (B. Pullman, P. O. P. Ts'o, and H. Gelboin, eds.), pp. 143-156. D. Reidel Publishing Co., Boston, MA.

136. Thakker, D. R., Yagi, H., and Jerina, D. M. (1978). Analysis of polycyclic aromatic hydrocarbons and their metabolites by high-pressure liquid chromatography. In "Methods in Enzymology," (S. Fleischer and L. Packer, eds.), Vol. LII, pp. 279-296. Academic Press, New York.

137. Zacharias, D. E., Glusker, J. P., Fu, P. P., and Harvey, R. G. (1979). Molecular structures of the dihydrodiols and diol epoxides of carcinogenic polycyclic aromatic hydrocarbons. X-ray crystallographic and NMR analysis. J. Am. Chem. Soc. 101, 4043-4051.

138. Lehr, R. E., Schaefer-Ridder, M., and Jerina, D.M (1977). Synthesis and properties of the vicinal trans dihydrodiols of anthracene, phenanthrene, and benzo[a]anthracene. J. Org. Chem. 42, 736-744.

139. Sims, P., and Grover, P. L. (1981). Involvement of dihydrodiols and diol epoxides in the metabolic activation of polycyclic

hydrocarbons other than benzo[a]pyrene. In "Polycyclic Hydrocarbons and Cancer" (H. V. Gelboin and P. O. P. Ts'o), Vol. 3, pp. 117-181. Academic Press, New York, and references therein.

140. Rogan, E. G., Roth, R. W., Katomski-Beck, P. A., Laubscher, J. R., Cavalieri, E. L. (1980). Non-enzymatic ATP-mediated binding of hydroxymethyl derivatives of aromatic hydrocarbons to DNA. Chem.-Biol. Interactions 31, 51-63.

141. Flesher, J. W. and Sydnor, K. L. (1973). Possible role of 6-hydroxymethylbenzo[a]pyrene as a proximate carcinogen of benzo[a]pyrene and 6-methylbenzo[a]pyrene. Int. J. Cancer 11, 433-437.

142. Dewhurst, F., Kitchen, D. A., and Calcutt, G. (1972). The carcinogenicity of some 6-substituted benzo[a]pyrene derivatives in mice. Br. J. Cancer 26, 506-508.

143. Tay, L. K., Sydnor, K. L., and Flesher, J. W. (1979). Binding of 6-hydroxymethylbenzo[a]pyrene and 6-acetoxymethylbenzo[a]-pyrene to DNA. Chem.-Biol. Interactions 25, 35-44.

144. Watabe, T., Ishizuka, T., Isobe, M., and Ozawa, W. (1982). A 7-hydroxymethyl sulfate ester as an active metabolite of 7,12-dimethylbenz[a]anthracene. Science 215, 403-405.

145. Fieser, L. F., and Hershberg, E. B. (1938). The oxidation of methylcholanthrene and 3,4-benzpyrene with lead tetraacetate; further derivatives of 3,4-benzpyrene. J. Amer. Chem. Soc. 60, 2542-2548.

146. Minlon-Huang (1946). Simple modification of the Wolff-Kishner reduction. J. Amer. Chem. Soc. 68, 2487-2488.

147. Dewhurst, F., and Kitchen, D. A. (1972). Synthesis and properties of 6-substituted benzo[a]pyrene derivatives. J. C. S. Perkins I, 710-712.

148. Chou, M. W. and Yang, S. K. (1979). Combined reversed-phase and normal-phase high-performance liquid chromatography in the purification and identification of 7,12-dimethylbenz[a]-anthracene metabolites. J. Chrom. 185, 635-654.

149. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

150. Omura, T., and Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370-2378.

151. Nebert, D. W. and Gelboin, H. V. (1968). Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I.

Assay and properties of induced enzyme. J. Biol. Chem. 243, 6242-6249.

152. Wong, T. K., Chiu, P.-L., Fu, P. P., and Yang, S. K. (1981). Metabolic study of 7-methylbenzo[a]pyrene with rat liver microsomes: separation by reversed-phase and normal-phase high performance liquid chromatography and characterization of metabolites. Chem.-Biol. Interactions 36, 153-166.

153. Hamernik, K. L., Chiu, P.-L., Chou, M. W., Fu, P. P., and Yang, S. K. (1983). Metabolic activation of 6-methylbenzo[a]pyrene. In "Polynuclear Aromatic Hydrocarbons: Formation, Metabolism, and Measurement" (M. Cooke and A. J. Dennis, eds.), pp. 583-597. Battelle Press, Ohio.

154. Morrison, R. T. and Boyd, R. N. (1983). "Organic Chemistry," Fourth Edition, pp. 970-972. Allyn and Bacon, Inc., Boston.

155. Levin, W., Alvares, A., Jacobson, M., and Kuntzman, R. (1969). Effect of storage of frozen liver microsomal preparations on the hydroxylation of testosterone and phenobarbital and the N-demethylation of ethylmorphine. Biochem. Pharmacol. 18, 883-889.

156. Chiu, P.-L., Weems, H. B., Wong, T. K., Fu, P. P., and Yang, S. K. (1983). Stereoselective metabolism of benzo[a]pyrene and 7-methylbenzo[a]pyrene by liver microsomes from sprague-dawley rats pretreated with polychlorinated biphenyls. Chem.-Biol. Interactions 44, 155-168.

157. Jerina, D. M., Selander, H., Yagi, H., Wells, M. C., Davey, J. F., Mahadevan, V., and Gibson, D. T. (1976). Dihydrodiols from anthracene and phenanthrene. J. Amer. Chem. Soc. 98, 5988-5996.

158. Buhler, D. R., Figen, U., Thakker, D. R., Slaga, T. J., Conney, A. H., Wood, A. W., Chang, R. L., Levin, W., Jerina, D. M. (1983). Effect of a 6-fluoro substituent on the metabolism and biological activity of benzo[a]pyrene. Can. Res. 43, 1541-1549 and references therein.

159. Lehr, R. E., Kumar, S., Levin, W., Wood, A. W., Chang, R. L., Buening, M. K., Conney, A. H., Whalen, D. L., Thakker, D. R., Yagi, H., and Jerina, D. M. (1980). Benzo[e]pyrene dihydrodiols and diol epoxides: Chemistry, mutagenicity and tumorigenicity. In "Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects" (A. Bjørseth and A. J. Dennis, eds.), pp. 675-688. Battelle Press, Ohio.

160. Lehr, R. E., Taylor, C. W., Kumar, S., Levin, W., Chang, R., Wood, A. W., Conney, A. H., Thakker, D. R., Yagi, H., Mah, H. D., and Jerina, D. M. (1979). Differences in metabolism provide a basis for the low mutagenicity and carcinogenicity of benzo[e]pyrene compared to benzo[a]pyrene. In "Polycyclic

Aromatic Hydrocarbons" (P. W. Jones and P. Leber, eds.), pp. 37-49. Ann Arbor Science Publishers, Ann Arbor, MI.

161. Chiu, P.-L., Fu, P. P., and Yang, S. K. (1982). Effect of a peri fluoro substituent on the conformation of dihydrodiol derivatives of polycyclic aromatic hydrocarbons. Biochem. Biophys. Res. Commun. 106, 1405-1411.

162. Fu, P. P., and Yang, S. K. (1982). Stereoselective metabolism of 6-bromobenzo[a]pyrene by rat liver microsomes: absolute configuration of trans-dihydrodiol metabolites. Biochem. Biophys. Res. Commun. 109, 927-934.

163. Buening, M. K., Levin, W., Karle, J. M., Yagi, H., Jerina, D. M., and Conney, A. H. (1979). Tumorigenicity of bay-region epoxides and other derivatives of chrysene and phenanthrene in newborn mice. Can. Res. 39, 5063-5068.

164. Chang, R. L., Levin, W., Wood, A. W., Lehr, R. E., Kumar, S., Yagi, H., Jerina, D. M., and Conney, A. H. (1981). Tumorigenicity of the diastereomeric bay-region benzo[e]pyrene 9,10-diol-11,12-epoxides in newborn mice. Can. Res. 41, 915-918.

165. Wislocki, P. G., Buening, M. K., Levin, W., Lehr, R. E., Thakker, D. R., Jerina, D. M., and Conney, A. H. (1979). Tumorigenicity of the diastereomeric benz[a]anthracene 3,4-diol-1,2-epoxides and the (+)- and (-)-enantiomers of benz[a]anthracene 3,4-dihydrodiol in newborn mice. J. Natl. Cancer Inst. 63, 201-204.

166. Wood, A. W., Chang, R. L., Huang, M.-T., Levin, W., Lehr, R. E., Kumar, S., Thakker, D. R., Yagi, H., Jerina, D. M., and Conney, A. H. (1980). Mutagenicity of benzo[e]pyrene and triphenylene tetrahydroepoxides and diol-epoxides in bacterial and mammalian cells. Can. Res. 40, 1985-1989.

167. The absolute configuration of the major trans 9,10-dihydrodiol enantiomer formed from benzo[a]pyrene metabolism was reported to be [9R,10R] by Dr. D. M. Jerina at the Fifth International Symposium on Microsomes and Drug Oxidations, July 26-29, 1981, Tokyo, Japan.

168. Kedzierski, B., Thakker, D. R., Armstrong, R. N., and Jerina, D. M. (1981). Absolute configuration of the K-region 4,5-dihydrodiols and 4,5-oxide of benzo[a]pyrene. Tetrahedron Lett. 22, 405-408.

169. Yagi, H., Akagi, H., Thakker, D. R., Mah, H. D., Koreeda, M., and Jerina, D. M. (1977). Absolute stereochemistry of the highly mutagenic 7,8-diol-9,10-epoxides derived from the potent carcinogen trans-7,8-dihydroxy-7,8-dihydrobenz[a]pyrene. J. Am. Chem. Soc. 99, 2358-2359.

170. Wislocki, P. G., Roth, R. W., Saugier, R. L., Johnson, S. L., Rogan, E. G., and Cavalieri, E. G. (in press). Carcinogenesis.

171. Alvares, A. P. and Kappas, A. (1977). Heterogeneity of cytochrome P-450s induced by polychlorinated biphenyls. J. Biolog. Chem. 252, 6373-6378.